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CONTENTS.

PHYSIOLOGY AND PHARMACOLOGY.

CHEMISTRY.

EXPERIMENTAL SURGERY.

EXPERIMENTAL BIOLOGY.

THE HOSPITAL OF THE ROCKEFELLER INSTITUTE.

ANIMAL PATHOLOGY.

TORULA INFECTION IN MAN.

A Group of Cases, Characterized by Chronic Lesions of the Central Nervous System, with Clinical Symptoms Suggestive of Cerebral Tumor, Produced by an Organism Belonging to the Torula Group (Torula histolytica, N. Sp.).*

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PLATES 1 TO 9.

I. INTRODUCTION.

Two cases from the clinic of Dr. Harvey Cushing, at the Peter Bent Brigham Hospital, presented at autopsy unusual lesions in the brain and meninges. The histories and physical examinations included such signs of cerebral tumor as to indicate decompression or exploratory operation, but the pathological examination in each case proved the complete absence of tumor. Lesions were found, however, which fully explained the clinical symptoms and physical signs. Enormous numbers of organisms having many points of resemblance to those of blastomycosis occurred in all of the lesions in such a manner as to leave no doubt of their causal relations. Certain differences from the usual descriptions of the organisms of the blastomycosis group and their lesions made a careful study necessary to determine the relations of our cases.

Two problems which have received increasing attention in late years, without great progress toward their solution, were thus brought to our notice by the study of the cases,—the problem of cerebral pseudotumor, and the problem of the relationships of the lower fungi forming the group called blastomycosis. It seemed probable that our cases might throw light upon both these questions.

In the study of the first problem, that of pseudotumor cerebri, the literature furnished a considerable number of cases in which symptoms and signs of brain tumor existed for a short or long time,

with subsequent recovery, or with indefinite autopsy findings. The cases were mysterious, but many reports by competent observers left no doubt of their existence. No well known disease could explain them; the promise of their solution lay in the finding of similar cases fully studied and made clear, or in the investigation of little known disease capable of producing such syndromes. To show that our cases and experiments suggest a solution is part of the object of this paper.

In trying to solve the other problem, that of the relationships of the organisms of the blastomycosis group, we studied the literature and made animal experiments. We used in our experiments pure cultures of three different organisms: one isolated from a human case of cutaneous blastomycosis, one from a human case of coccidioidal granuloma, and one from Dr. Frothingham's case of torula infection in a horse. Inoculations were made in various ways upon several species of laboratory animals, and agglutination experiments done upon the infected animals. We realized that a great deal of work had been done upon the cultural characteristics of these organisms, but hoped that the careful study of the histological changes and the serological reactions would give more definite information as to the similarity or identity of the diseases produced.

Wolbach says, in regard to the blastomycotic group of organisms:

For the present we wish to emphasize the necessity for careful studies upon the organisms by means of animal experiments, for it is by this method only that the more striking differences between the types may be demonstrated. Cultural methods as applied to bacteria have not been of value to us.

It is difficult, and often impossible, to make fungi produce a characteristic fructification. None has yet been discovered for the group of organisms causing the cases of blastomycosis localized near Chicago.

In our second case cultures of the ventricular fluid made in bouillon were injected into mice intraperitoneally, and at autopsy a meningitis was found with organisms like those in the human meninges. In the first case no spinal fluid was obtained on account of the danger of puncture during pressure symptoms, and the technique of formalin injection of the brain before removal prevented cultures at autopsy.

The lesions were so peculiar, however, that when we succeeded in reproducing them in animals no doubt remained of their identity with the experimental ones.

As the study of our cases and experimental results progressed, we found that another problem of importance came into relation with our work. It is recognized that lesions similar to those of tuberculosis occur in other diseases; coccidioidal granuloma, for instance, has often very similar lesions, but the parasite is so large that its recognition is not difficult; blastomycosis occasionally produces tubercle-like nodules; other fungi are known to produce reactions more or less closely similar to tuberculosis. We found in our cases and in our experimental work lesions so closely resembling tuberculosis that a differential diagnosis would be impossible without finding the parasites. The parasites are so small that they could easily be overlooked, and in the later stages of the lesions they disappear entirely, leaving as a result tubercle-like nodules with no trace of their origin. The lesions have typical caseation or consist of miliary nodules without caseation. A number of human cases with similar lesions in which no tubercle bacilli could be found have come to our attention in this hospital. It is possible that many cases diagnosed as tuberculosis may be due to this organism.

II. HISTORICAL.

An enormous mass of literature has grown up with reference to lesions in human beings, produced by organisms which bud without producing mycelium in the tissues, and by some that are roughly similar, but that do not bud in the tissues. All of these have been loosely classified as cases of blastomycosis. As many observers long ago realized, there is nothing distinctive about the budding type of growth, for many fungi higher than yeasts have a stage in which they reproduce by budding.

1. Evidence That Coccidioidal Granuloma Is Distinct from the Other Diseases Called Blastomycosis.

It seemed possible, therefore, that the name blastomycosis would eventually be applied to a group of diseases produced by various organisms. This has proved to be the case, for at least two distinct types have been separated by different observers. They are the coccidioidal granuloma and the blastomycosis types. The difference is simple; the coccidioidal organism never buds in

tissue but sporulates; the blastomycotic organism buds but never sporulates. It is obvious that coccidioidal granuloma should never have been confused with blastomycosis. There are further differences. Thus the coccidioidal organism varies more in size, having large forms up to 30 to 40μ in diameter, which produce many small ascospores which escape from the capsule. The coccidioidal organism, furthermore, produces lesions more nearly like tuberculosis, and the infection more often spreads by the lymphatics as well as by the blood. The disease is almost invariably fatal (Wolbach, however, reports a case with recovery) and occurs practically always in males who have lived in the San Joaquin valley, California. Cases of blastomycosis have a wider distribution, the systemic cases recover in 10 per cent of the instances recorded, and cutaneous cases frequently recover without internal lesions. The patients are benefited, as a rule, by iodides, which have no effect on coccidioidal granuloma.

Among German observers, Buschke does not recognize a difference between the coccidioidal and the blastomycotic type, and confuses coccidioidal granuloma with coccidiosis. Wright regards them as different manifestations of the same disease. Ever since the first observations by Posadas and Wernicke, and Rixford and Gilchrist, evidence has accumulated that the diseases are really distinct. Wolbach accurately described the life cycle of the organism of dermatitis coccidioides, and found it entirely different from that of blastomycosis; MacNeal and Taylor studied the life cycle of the coccidioides organism, summarized the known cases, and concluded that the two diseases were distinct clinically, pathologically, and biologically. Other summaries of the literature by Ophüls, Ryfkogel, and Hektoen lead to the same conclusion. Innumerable studies of the organism of blastomycosis have never revealed the methods of reproduction by endosporulation characteristic of the coccidioidal type. A possible source of confusion in the early accounts was the occurrence, in apposition, of small forms in coccidioidal granuloma, which simulated buds. True budding is not found in coccidioidal granuloma, but is constant in blastomycosis. A recent paper by Brown and Cummins again establishes the differences between the two diseases and adds evidence from animal experiments.

The first study of our cases with the many budding forms showed that the picture was entirely different from that of coccidioidal granuloma. Our experiments substantiated the statements made by the other workers as to the differences in the two diseases, and showed us that the brain lesions we produced with *Coccidioides immitis* were different in type from those of our cases.

2. Previous Attempts to Classify the Blastomycoses.

Of the remaining group of true blastomycoses, many attempts by American workers to make divisions have resulted in indefinite conclusions. Ricketts, in his extensive study of organisms from seventeen cases of cutaneous blastomycosis, made a tentative division into blastomycetoid, or organisms showing a predominating tendency to bud, oidiomycetoid, or those showing a tendency to

bud or produce mycelium, and hyphomycetoid, or organisms tending to produce aerial hyphæ. The differences were somewhat inconstant and of degree rather than of kind. Hamburger studied organ'sms from four cases of systemic infection, and found them practically identical. Montgomery and Ormsby have asserted that a'l types hitherto described have been observed at different times in the same strain. Stober states that in his study of eleven strains the chief difference was in the size of mycelium and spores. In all the organisms studied myce'ium production has occurred sooner or later; it is favored by room temperature, dryness of the medium, and transplantation, while budding occurs chiefly when there is abundant moisture and a thermostat temperature.

Of German workers, Buschke divides the blastomycoses into diseases produced by true yeasts, saccharomycoses, and those produced by organisms which bud and produce mycelium, the oidiomycoses. Two cases of true yeast infection in man are sufficiently well established to deserve attention.

When we turn to botanical literature we find that there is a distinction between the true yeasts and the torulæ (or yeast-like organisms which do not produce endospores) on the one hand, and the oidium group on the other hand, in which both budding and hypha production occur. The organisms of ordinary blastomycosis evidently belong to the oidium group.

In animal experiments with lower fungi, it was observed that the torulæ had marked pathogenic powers for animals,—greater than those of yeasts   Rabinowitsch, in an investigation of forty specics of yeast, found only eight pathogenic. Klein found a pathogenic torula in milk, and produced lesions in animals with a pure culture; Cohn worked with the same organisms and obtained similar results. Nichols, in his experiments upon budding organisms in relation to cancer, used two strains of torula and easily produced lesions. Many other workers have confirmed these statements. Often a tendency for the organisms to produce brain lesions is noted. Frothingham found a tórula naturally producing lesions in a horse, and in animal experiments proved its marked pathogenic powers. It was possible, then, that an organism pathogenic for animals would sooner or later be found to produce disease in man. We could not find, however, cases reported as torula infection.

In studying the organisms of the cases of b'astomycosis, it seemed of value to look up the literature, first from the po'nt of view of brain lesions, and to tabulate all the cases classified as blastomycosis which involved the brain, and then to look for cases like ours and for atypical cases; secondly, to tabulate the cases of systemic blastomycosis without brain lesions, and observe similarities and differences.

The results of our tabulations show that the cases of systemic blastomycosis without brain lesions are closely alike clinically and pathologically, but there are divisions to be made within this group, as will be' explained in detail in the discussion of the cases.

The cases with brain lesions, on the contrary, are a much less homogeneous group. A number of cases stand out from the rest as distinctly atypical, but similar to each other.

3. Cases of Systemic Blastomycosis with Brain Lesions.

A. Abstracts of Ten Cases.

Case 1.—Reported by von Hansemann.

Patient.—A laborer, 18 years old, with tuberculosis of the lungs (bacilli in sputum).

Present Illness.—Onset with apathy. Double abducens paralysis, vertical nystagmus, unequal pupils, choked disc, slow pulse (46). Spinal puncture gave at first a watery, later a slightly cloudy fluid, in which were at first few, then more numerous lymphocytes, but always peculiar large round bodies considered to be corpora amylacea. They were in part singly, in part doubly contoured, of very variable size, some larger, some smaller than an erythrocyte, were colorless and moderately refractive. There were also biscuit forms, and longer forms in three to four jointed figures that recalled myelin drops. Cultures did not grow.

Diagnosis.—Tubercular meningitis.

Course.—Then followed vomiting, involuntary discharge of feces and urine, delirium, and coma, and death 19 days after entrance.

Autopsy.—Tuberculosis of the lungs. The pia and arachnoid were saturated and the ventricles filled with a slightly cloudy fluid containing bodies similar to those described above. On the surface of the brain were about sixty small cysts, the largest the size of a hemp-seed, only slightly projecting above the surface, and on section emptying themselves partially, leaving slimy colloidal masses. Section showed similar cysts, often confluent, especially numerous in the corpus striatum and its neighborhood. The corpus striatum and part of the thalamus bulged out into the lateral ventricle with a myxomatous-like mass. Cultures did not grow.

Microscopical Examination.—Cysts and tumor-like masses consisted of enormous collections of yeast-like bodies embedded in a colloid-like substance, and the surrounding brain substance showed very little reaction. In many colonies were numerous large phagocytic cells, but for the most part the organisms lay free. In the pia was a slight increase in lymphocytes and leukocytes, especially the former, but the numerous organisms called forth almost no reaction of the meningeal tissues.

Case 2.—Reported by Türck.

Patient.—A woman, 43 years old, servant, entered the hospital on Apr. 21, 1906.

Past History.—Scarlet fever in childhood; epilepsy with general convulsions since the age of 4 years; operation for purulent neck glands at 11; later an operation for eye trouble. In recent years much coughing, but no hemoptysis.

Present Illness.—A sudden onset at the end of March, 1906, with headache, vomiting, and a chill. There followed weakness, frequent nocturnal or early morning vomiting, and increasing stiffness of the neck.

Examination.—Apr. 21. A slight, poorly nourished woman; temperature 38° to 38.2°C. Frontal and occipital headache; cranial tenderness, and tenderness of the stiff cervical, thoracic, and lumbar spinal muscles; general hyperesthesia toward pin pricks, but no sensitiveness of the abdominal or leg muscles; positive Kernig; normal knee jerk; throat normal; scars on neck; palpable glands in the neck and axilla; lungs with dulness at the apices, with râles and bronchial breathing; no sputum; urine with much nucleo-albumin and mucin, and a trace of serum albumin.

Course.—No marked change. No cranial nerve involvement. Temperature irregular, 37° to 38°C., pulse 80. Lumbar puncture on Apr. 25 gave a clear serous fluid under increased pressure. At the end of 4 weeks the condition of the patient was the same as at entrance. This caused doubt as to the diagnosis of tubercular meningitis, which was made on entrance. A second lumbar puncture on May 3 gave 5 cc. under high pressure. The fluid was slightly cloudy.

Microscopical Examination.—Many typical yeasts of round or oval shape. The extraordinary finding made a verification necessary and a second puncture was done with especial care on May 5. The specific gravity of the fluid was 1,008. A slight clot formed on standing. No tubercle bacilli were found, but there were enormous numbers of organisms often joined as are yeast cells, numerous small lymphocytes, rare red cells, and groups of large phagocytic mononuclear cells. On May 8 examination showed great emaciation; otherwise no change.

Blood Examination.—Red cells 5,210,000; hemoglobin 14.49 per cent (Fleischer-Miescher); white cells 7,000; increased blood platelets.

Differential Count.—Polymorphonuclears 91.5 per cent; lymphocytes 3.6 per cent; large mononuclears 4.9 per cent.

On May 10 an increasing white coat over the throat was noticed. The general condition grew worse. Lumbar punctures on May 13, 14, and 15 gave results similar to the earlier ones. On May 15 the temperature rose to 40.1°C. and the patient died.

Autopsy.—Dura thin and smooth; a soft brain with slightly cloudy hydrocephalus, and cloudy edema of the meninges of the Sylvian fissure. The mucous membranes of the tongue, throat, soft palate, and esophagus thickened in increasing degree to the cardia, with a crumbly, dry, yellowish white layer. The lung apices were thick with caseous nodules and cavities. A few small caseous mesenteric glands. Liver capsule thickened. A group of enlarged glands near the aorta.

Microscopical Examination.—Organisms in the ventricular fluid, but none in the brain substance, or in the ependyma or choroid plexus. Large numbers of these in the meninges, a few within cells, but with no reaction of tissues, except occasional collections of lymphocytes. No organisms were seen in the vessels or vessel walls. The esophagus showed loss of epithelium, with a fibrinous deposit; joined organisms penetrated even to the striated muscle beneath. No

mycelium was found. Cultures from the throat gave organisms similar to those from the meninges, with no mycelium formation.

Organisms.—The organisms in the lumbar puncture were found to be elliptical or ovoid, and equal in size to a red blood cell or slightly smaller. They almost always had typical yeast joinings to the number of two to four elements. The long diameter varied from 3.33 to 8.33μ, the width from 2.2 to 6.66μ. The small ones were usually ovoid; the large ones approached a spherical form, and often had a double contour. One or two poorly marked eccentric oval nuclei were present, and one to eight refractive droplets. Many giant forms of from 10 to 15μ were found, which were more refractive, with larger oil droplets, a double contour, frequent yeast joinings, and a Gram-negative capsule. The phagocytized cells showed a clear capsule almost equal in width to the yeast cell.

Cultures.—Mycelium formation was never found. The yeast grew least well on bouillon, gelatin, and agar. Growth appeared in 4 to 5 days and then proceeded slowly. No liquefaction of the gelatin took place. Glucose was not fermented. The best growth was obtained on glucose-agar. No spore production was noted.

The atrium of infection was considered to be the throat.

Case 3.—Reported by Eisendrath and Ormsby. Final report by Le Count and Myers.

Patient.—A Polish laborer, 33 years old.

Illness.—Discomfort in the right chest for 6 months, then numerous cutaneous lesions, followed in 4 months by edema of the face and extremities, muscular weakness, emaciation, pallor, and elevated temperature. Shortly before death incontinence of urine and feces was frequent. Death occurred during a convulsion, after 30 months' illness.

Autopsy.—Blastomycotic bronchopneumonia; blastomycosis of the peribronchial lymph nodes, of the pleura, the subpleural and retropharyngeal tissue, the liver, kidneys, colon, spinal column, external spinal dura, cerebellum, left elbow, both knee and ankle joints, skin, and subcutaneous tissues. Emaciation.

Cerebellum.—On horizontal section nearly the entire external half of the right lobe was involved in coalescent areas of softening with wide margins of necrotic tissue in which small nodules were present.

Microscopical Examination.—Small areas of necrosis surrounded by granulation tissue and inflamed cerebellar tissue. The margins of the lesion were made irregular by very minute abscesses, some no larger than a fat cell, containing mono- and polymorphonuclear leukocytes and organisms multiplying by budding. Giant cells containing organisms were found at the periphery of the larger lesions. The granulation tissue often occurred in peculiar masses, containing many cells of chronic inflammation, and very small non-budding blastomycetes. Several large empty capsules were found, with numerous small forms near by. Intact large forms contained several granules resembling nucleoli, with pale areas oc-

casionally visible about them. The small forms each contained one granule. These appearances to the author indicated endosporulation.

Cultures.—Cultures from the cutaneous lesions and the sputum gave spherical and oval organisms, much fine and coarse mycelium, later conidia and aerial hyphæ. Guinea pigs inoculated with the material developed local lesions.

Case 4.—Reported by Krost, Stober, and Moes.

Patient.—A Pole, 42 years old, resident in the United States for 4 years.

Illness.—Pulmonary symptoms, then skin lesions. No brain symptoms were noted except moroseness and irritability toward the end. Blood cultures positive for blastomycetes. Duration of illness, 6 months.

Autopsy.—Miliary and nodular blastomycosis of the lungs, kidneys, spleen, cerebrum, pleura, and lymph nodes. Multiple blastomycotic abscesses of the cerebrum, cerebellum, prostate, pleura, skin, and osseous, muscular, and cutaneous tissue. Parenchymatous nephritis. Fatty changes, adenoma, and angioma of the liver. General lymphatic hyperplasia. Atrophy of the testicles.

Brain.—Weight 1,400 gm. No gross lesions externally. On section, an abscess 2 mm. in diameter was found in the cortex near the middle of the parieto-occipital sulcus. A small nodule was found in the right parietal lobe, one in the right temporal lobe, and two in the occipital lobe; the nodules were in the cortex, usually at the margin of the white matter. There was a nodule in each lobe of the cerebellum.

Microscopical Examination.—There was much necrosis. "Apparent bands of fibrous tissue between adjacent lesions, due to pressure," and numerous "bizarre" giant cells. Blastomycetes were present in the lesions.

Case 5.—Reported by Lewison and Jackson.

Patient.—An Italian, 17 years old, resident in the United States for 4 years.

Illness.—1 year's duration. No nervous symptoms noted.

Autopsy.—Indurated blastomycotic bronchopneumonia of the left lung, blastomycosis of the bones, subcutaneous tissues, pons varolii, inguinal and axillary lymph nodes; acute vegetative mitral endocarditis, fatty liver, parenchymatous degeneration of the kidneys, hyperplasia of the lymphoid tissue of the ileum, edema of the lungs, hydropericardium, atrophy of the heart, pancreas, and testes, emaciation, and anemia. There was one blastomycotic nodule in the brain, a few mm. below the ventral surface of the pons, containing numerous organisms, and apparently formed by the coalescence of small lesions.

No note as to histology.

Case 6.—Reported by Myers and Stober.

Patient.—American.

Illness.—The only symptoms referable to brain lesions were irritability toward the end, with occasional refusal of food and medicine, and delirium a few days before death.

Autopsy.—"Healing and discharging blastomycotic abscesses and ulcers of

the skin; blastomycotic abscesses of the bones and muscles; blastomycosis of the lungs, peribronchial lymph nodes, liver, spleen, pancreas, kidneys, cerebrum, and cerebellum; serofibrinous and fibrous pleuritis; parenchymatous nephritis; chronic splenitis; hyperplasia of the mesenteric lymph nodes, pigmentation of Peyer's patches; decubital ulcers; emaciation." The brain lesions consisted of abscesses, about some of which connective tissue walls were present.

Case 7.—Reported by Bechtel and Le Count.

Patient.—A Swede, 38 years old.

Illness.—8 months' duration.   No nervous symptoms noted.

Autopsy.—Ulcerative blastomycosis of the upper lobe of the left lung; disseminated blastomycosis of the lungs, liver, spleen, kidneys, adrenals, brain, subcutaneous tissues, and skin; blastomycotic caries of the left iliac and right parietal bones; fibrinous and fibrous pleurisy; hyperplasia of the tracheobronchial, cervical, and mesenteric lymph nodes; red marrow in the femur; emaciation; atrophy of the liver; fibrous mural endocarditis.

Brain.—Fourteen small lesions in the cerebrum, most of them located at the bottom of sulci or at the junction of gray and white matter, a few in the basal ganglia and deeper parts, varying from 2 to 4 mm. in diameter.   Two larger lesions in the cerebellum, one 15 by 11 mm. in the white substance between the dentate nuclei and slightly above them, and one 4 mm. posteriorly.

Microscopical Examination.—The histological note states only that there was but little tissue reaction about the lesions.

Case 8.—Reported by Riley and Le Count.

Patient.—An Italian laborer, 31 years old.

Illness.—After an illness of 7 months the first nervous symptoms were noted a few days before death as neck rigidity, slight strabismus, exaggerated reflexes, and a positive Babinski.

Autopsy.—Chronic blastomycosis of the upper lobe of the left lung; erosion of the third and fourth thoracic vertebræ; blastomycotic abscesses of the root of the right lung; disseminated miliary blastomycosis of the spleen and liver; blastomycotic basilar meningitis; abscess of the prostate, peritonsillar tissue, epididymis, and skin (with ulcers), and subcutaneous tissues; caries of the foot bones; multiple intercostal subpleural abscesses; blastomycotic tracheobronchial lymphadenitis; fibrinous and fibrous pleuritis; fibrous peritonitis; emaciation and anemia; brown atrophy of the heart; atrophy of the liver; slight sclerosis of the aorta and coronaries; emphysema of the lungs; ossification of the thyroid cartilage.

Brain.—A thick gray pus or fibrinopurulent exudate covered the base of the brain, thickest over the circle of Willis.   Aggregated masses of small size were found in the fundi of the sulci, especially on the right, at times forming marble-sized lesions.   No deep lesions were found.

No note as to histology.

Cultures.—Cultures from the cerebrospinal fluid gave a pure growth of blastomycetes.

Organisms in the last five cases were all described in a common cultural report, a partial summary of which follows. In the tissues, discharges, and pus they appeared in pairs or clumps as round or oval bodies, with frequent budding. The size varied from 3 to 30μ, with 20μ as the average size. There was an outer refractile capsule and a clear central portion, the capsule varying in thickness with the size of the organism, and occasionally being invisible in the organisms in old foci. The capsule rarely stained. The central portion was granular and took basic stains, and often contained three to ten spherical basophilic granules. No hyphæ were found in the tissues, and endosporulation was suggested only in Case 3. In cultures there was first a proliferation by budding, then hypha production, and finally a formation of aerial hyphæ. Occasionally segmentation of the mycelium into spores was seen. Another slightly different type had a very fine mycelium and small spores.

Case 9.—Reported by Rusk.

Past History.—Obscure. Previous history of "rheumatism."

Examination.—A confused and talkative patient, becoming exhausted, semistuporous, and unintelligent. Pupils irregular and reactive; knee jerks, Achilles jerks, and abdominal reflex absent; diminished pharyngeal reflex; muscular weakness; general diminished sensibility to touch and pain with preservation of temperature sense; some tenderness on pressure over nerve trunks; fine tremor of tongue, coarse tremor of extremities. Temperature 98°F., pulse 108, respiration 26. Stupor.

Course.—Stupor increased, and after an attack of hematuria the patient died. Duration of observed course about 30 days.

Diagnosis.—Senile dementia.

Autopsy. Brain.—Cerebrospinal fluid not cloudy. Frontal convolutions moderately atrophied. The pia was diffusely hazy.

Microscopical Examination.—A chronic inflammatory reaction in the meninges, over the base, along the Sylvian fissure, over the cerebellum and spinal cord, with infiltration of lymphoid, plasma, and large endothelial cells. Giant cells with peripheral nuclei and a circumscribed border were found. Organisms were frequently enclosed in giant cells or endothelioid cells. There were no areas of caseation and no miliary abscesses. The neuroglia of the first layer of the cortex showed a diffuse and general hyperplasia, with increased fibrils and cells. No alterations were found in the nerve cells. In the cortex were two kinds of lesions: first, extensions from the pial lesions; second, circumscribed areas in the basal ganglia, about 1 cm. in diameter, in which the tissue appeared forced apart and filled with gelatinous material. There was a scanty reaction about these lesions of the same type as in the pia.

Lungs.—The left lung contained a group of cavities from the limits of visibility to 2 cm. in diameter, filled with a sticky gelatinous material. Laterally from them was a firm triangular area containing tiny pale yellow foci. Sections from the gelatinous masses showed them to be composed of a spherical, doubly

contoured organism, with a sticky, jelly-like capsule of varying thickness, lying in an amorphous matrix. The organism proliferated by budding. The walls of the cavities were formed by fibrous tissue, apparently not newly formed, but the remnant of bronchi or alveoli, which the organisms appeared to be in the process of eroding; the lining cells were swollen, the protoplasm was finely vacuolated, and the nuclei were pyknotic. Some little distance further in the tissue the first evidence of inflammatory reaction occurred, consisting of a slight accumulation of lymphoid and plasma cells, with a few multinucleated cells.

Sections from the firm tissue showed a diffuse chronic granulomatous process with large giant cells containing the organisms. No caseation or interstitial abscess formation was present. Organisms were scattered in the tissue, in cells, and free. Occasional small hemorrhages were present.

No lesions were found in other organs.

Organisms.—Organisms were present in the pia in giant cells, or free singly, or in zoogleal masses. Spherical organisms with a gelatinous capsule, proliferating by budding, filled the lung cavities and were seen in giant cells in the granulation tissue. No cultures were made.

Case 10.—Reported by Rusk.

Patient.—German, 57 years old.

Past History.—Positive for lues.

Illness.—Weakness, morning expectoration, painful muscles, and shortness of breath, were followed by transitory hemiplegias and syncopal attacks, and a temporary loss of speech. Later there were times of confusion and forgetfulness of decencies of dress.

Examination.—Frontal headache and abdominal pain were the complaints. The man was depressed, agitated, perplexed, unable to concentrate his attention, disoriented for time, place, and person; had impaired memory, lack of interest, unstable emotions, poor enunciation with slurring and transpositions; he realized that he was sick, and had no delusions or grandiose ideas. His pupils failed to react to light, the knee and Achilles jerks were absent, the pharyngeal reflex was active. There were ataxia, a positive Romberg sign, a coarse tremor of the face and tongue, diminution in the acuteness of pain and touch over the entire surface of the body with differentiation of temperature. Temperature 101.8°F., pulse 95, respiration 21.

Spinal Fluid.—Positive Noguchi reaction; marked lymphocytosis.

Course.—Increased weakness; urinary retention; death after 24 hours' stupor. Total illness 2 years' duration; nervous symptoms for 10 months.

Clinical Diagnosis.—General paresis, tabetic type.

Autopsy.—Pial haziness and adhesions; cloudy cerebrospinal fluid in the lower part of the spinal canal; a few granulations in the fourth ventricle.

Brain.—Slightly atrophic; weight 1,226 gm.

Microscopical Examination.—The reaction was of the same type as in the previous case with the addition of a few poorly defined miliary abscesses and rare

areas with necrotic caseated centers. The meningitis was distributed over the whole cortex. A few small arteries had a subintimal infiltration. Giant cells of enormous size were noted. Organisms were found throughout the pia. There were similar extensions into the cortex, and the same glial reaction. The lungs showed a bronchopneumonia with occasional small bronchi containing parasites, and small granulomatous masses growing from the alveolar walls contained the organisms. Larger granulomatous masses had typical giant cells with the organisms. There was no caseation. Granulomatous lesions containing the organisms were found in the kidney. No cultures were obtained.

Organisms of the last two cases were spherical, doubly contoured, varying in size up to 20μ, with a homogeneous viscid capsule outside the wall, embedded in a homogeneous matrix; staining weakly, with no differentiation of internal structures; the capsule stained with hematoxylin; reproduction was by budding only. Naked forms were seen, free and within cells. Star-like forms occurred after Zenker mordanting.

B. Discussion of Cases.

a. Characteristics of Six Cases Similar to Those of the Usual Form of Systemic Blastomycosis and Cutaneous Blastomycosis.

All but four of these cases had either skin or subcutaneous tissue lesions and presented the usual picture of blastomycotic infection, clinically and in regard to the pathology and the characteristics of organisms in tissues and cultures. The brain involvement was part of a general infection, and clinically, at least, an unimportant part, for no symptoms were produced except in two cases shortly before death,—in Case 3, where the patient died during a convulsion, and in Case 8, in which signs of meningitis were manifested in the last days. We have studied the original reports of· all other reported cases of systemic blastomycosis, and have found that in all these there is involvement of the skin or subcutaneous tissues. Stober states that the most characteristic changes in systemic blastomycosis are cutaneous ulcerations, deep and superficial abscesses, and tubercle-like nodules in the viscera. In only three cases has the systemic involvement been the evident result of a spread from cutaneous lesions; in many cases infection in the bronchi and lungs constituted the primary focus, from which dissemination of organisms in the blood resulted in the skin and subcutaneous lesions. Thus a predilection is clear for the development of the blastomycetes in skin and subcutaneous tissues.

b. Four Cases Distinct Clinically, Pathologically, and Bacteriologically.

The four cases without skin or subcutaneous tissue lesions all have brain lesions of such a nature as to cause the predominating clinical symptoms throughout the disease.   In von Hansemann's case (Case 1) there were choked disc, slow pulse, unequal pupils, vertical nystagmus, double abducens paralysis, and death following vomiting, involuntary discharge of urine and feces, delirium, and coma.   In Türck's case there were headache and frequent vomiting, stiffness of the neck, sensory hyperesthesia, and a positive Kernig sign, the symptoms continuing for nearly 2 months.   Rusk's first case had confusion, semistupor, irregular pupils, absent knee jerks, and tremors. with death during stupor, 30 days after the first observation.   His second case had headache, depression, perplexity, impaired memory, transient hemiplegias, speech disturbance, Argyll Robertson pupils, absent knee and ankle jerks, ataxia, a positive Romberg, and coarse tremor. The diagnoses were as follows: Case 1, tubercular meningitis; Case 2, tubercular meningitis—ruled out later on account of the long duration; Case 9, senile dementia; Case 10, general paresis, tabetic type.

The marked difference in the character of the clinical histories of this group of cases suggests a careful study of the pathological findings in comparison with those of cases of blastomycosis showing skin or subcutaneous tissue lesions.   In Case 1 we find the brain lesions consisting of cysts composed of enormous collections of yeast-like bodies embedded in a gelatinous matrix, with very little reaction about them.   Numerous large phagocytic cells were in many colonies. In the meninges was an increase in lymphocytes and leukocytes. In the second case the brain lesions showed edema of the meninges, with large numbers of yeast-like bodies, a few of them within cells, with little reaction of the tissues except occasional collections of lymphocytes; the organisms in cells having a clear space about them equal to their diameter; in Cases 9 and 10 there were lesions in the brain consisting of enormous numbers of yeast-like cells with gelatinous capsules, embedded in a homogeneous matrix, with little or no reaction about them; naked forms occurred also; the meninges had a chronic inflammatory reaction with lymphoid, plasma, large endothe-

lioid cells, and giant cells. There was an increase in glia cells. In the second case there were similar lesions but so extensive as to result in areas of caseation. We have had an opportunity to see a section of a brain lesion from one of these cases, and were struck by the fact that the organisms did not produce the lesion by the pressure of their growth, but evidently by a solution of the brain tissue in their immediate neighborhood.

These pictures have no resemblance to those of the brain lesions of blastomycosis with skin lesions. Case 3 showed small areas of necrosis surrounded by granulation tissue and inflamed cerebellar tissue, with minute abscesses in the margins containing polynuclear and mononuclear leukocytes; Case 6, abscesses in the cerebellum and cerebrum, with connective tissue walls; Case 8, a thick purulent exudate over the base of the brain. In the other cases histological notes are very scanty and the assumption seems to be made that the lesions were much like those elsewhere; that is, with acute exudate and granulation tissue. Occasionally the reaction is slight in amount.

Thus the first group of cases is peculiar pathologically (1) in that in lesions within the brain substance there is produced a gelatinous or homogeneous substance in which the organisms are embedded, and about which there is a very slight chronic reaction; and (2) in that there are meningeal reactions of varying degrees of severity, but always containing only cells of chronic inflammatory type. In the cases of blastomycosis with skin or subcutaneous lesions, the brain lesions are described as abscesses with varying amounts of granulation tissue about them, or in one case as a purulent meningitis.

It is important to know whether lesions in other organs differed also. In Case 1 there were lesions of tuberculosis in the lungs, but no other lesions. Case 2 had a lesion in the esophagus with the same organisms as in the brain lesions, and caseous nodules and cavities in the lungs (due to tuberculosis?). There were no other lesions. Case 9 showed lesions in the lungs similar to those in the brain, with sticky gelatinous material and only a very slight chronic reaction; also areas of a chronic, non-caseating granulomatous process with large giant cells. Case 10 had a bronchopneumonia, and granulomatous lesions in the lungs and kidney like those in the previous case

Granulomatous lesions occur in ordinary blastomycosis, but the lung lesion in Case 9 is distinct. The freedom from extensive lesions in other organs in comparison with the usual cases of systemic blastomycosis, and the limited distribution of the lesions, are striking.

In the special group of cases the organisms produced the gelatinous material in three instances; in the second case there were no lesions in the brain, but wide clear zones occurred about the organisms when in cells. The organisms in Case 1 were not described fully; in Case 2 they were elliptical or ovoid, from 3.33 to 8.33μ in length, from 2.2 to 6.66μ in width, the large ones more nearly spherical, with a double contour. The interior contained only one or two eccentric oval masses and a few refractive droplets. Giant forms from 11 to 15μ were found. In Cases 9 and 10 the organisms were placed in a homogeneous matrix and had wide clear spaces or gelatinous capsules about them, varied in size up to 20μ, and had little or no differentiation of internal structure. In every case multiplication was by budding. No mycelium could be found in any of the cases, in tissue or in culture. In Cases 9 and 10 radiating projections from the cell wall were noted after certain stains. The walls of these organisms stained easily. These descriptions are markedly different from those of the other group of cases, in which the organism appears in tissue as a sphere from 10 to 15μ in diameter, with little or no space between it and other cells, but with a space between the cell wall and the central protoplasmic mass, which is finely granular and stains well, as a rule. The cell wall has poor staining qualities. No notes of gelatinous material in the brain lesions ever occur.

Cultures from the first case did not grow; from the second case cultures yielded organisms which did not produce mycelium or endospores even after long observations; from the ninth and tenth cases cultures were not obtained. Cultures of blastomycotic organisms sooner or later produce mycelium.

This group of cases then is distinct clinically, and pathologically from the cases of blastomycosis producing skin or subcutaneous tissue lesions.

In a search for other cases to be correlated with these we found the case described by Frothingham, through whose kindness we have seen sections and have procured tissues and cultures. Part of a

lobe of the right lung of a horse was enlarged, pinkish yellow, and gelatinous, microscopically showing a loose connective tissue network with budding organisms embedded in a homogeneous gelatinous matrix. The other organs of the horse were not examined. The organisms had for the most part a homogeneous non-granular interior, containing only a few fat droplets. The cellular reaction included varying numbers of large mononuclear cells, numerous giant cells, and some connective tissue. Pure cultures were obtained. They grew in 5 to 7 days, produced a slight amount of gelatinous material, multiplied only by budding, did not produce mycelium, or ferment dextrose, lactose, or saccharose, and did not form spores, even after observations of growth for 12 weeks on gypsum blocks at varying temperatures. They often formed large resting cells, and were facultative anaerobic. After subcutaneous inoculation they produced subcutaneous tissue lesions of the same type as those from which they were obtained. Thus this organism, which was thoroughly studied, was proved to be a torula, distinct from the oidium-like organisms of blastomycosis, and distinguished from true yeasts by the absence of spore production under special conditions.

The organisms found by Tokishige in lymphangitis epizootica of horses produced mycelium in culture; the disease resembled glanders, involving the skin, lymphatics, and respiratory mucous membranes. It has no relation to the case described by Frothingham.

The similarity of the sections of Frothingham's case to the group of cases described above was so great that a series of inoculations of pure cultures of the torula organism into animals was made in an attempt to produce brain lesions for comparison with those of the cases. The results will be given in detail later, but it may be stated here that lesions identical with the brain lesions of Cases 1, 2, 9, and 10 were produced in rats, thus adding to the evidence that these are true cases of torula infection.

4. Cases of Systemic Blastomycosis without Brain Lesions.

The study of the other cases of systemic blastomycosis without brain lesions led us to the conclusion that Buschke's cases of the true yeast infections formed a group distinct from either of the two just discussed. It is a small group; in fact only two undoubted cases can

be found.   Many cases reported as such, especially in dermatological literature, are incompletely reported, and of doubtful nature, as Buschke himself states.   The case of Busse and Buschke was so carefully observed by both, and the pathogenicity of the organism proved so conclusively by inoculations of a pure culture of the organism on the patient's skin, with the reproduction of the lesions, which contained a pure culture, that it can hardly be doubted.   The lesions were in the subcutaneous and cutaneous tissues, tibia, left ulna, and left sixth rib, with perirenal abscesses, and lesions in the lungs, kidneys, and spleen.   The organism had produced a purulent infiltration in the bones as in streptococcus lesions; in the skin there was overgrowth and destruction of epithelium with many giant cells. The lung contained abscesses bordered by fibrous tissue containing nodular accumulations of small round cells, in which were parasites. The parasites here were not often intracellular.   In the kidney was an abscess with acute interstitial inflammation.   The whole picture to Busse was that of a chronic pyemia.   The organism often had an adventitious capsule in tissues; in culture it fermented sugar and did not produce mycelium, even after long observation under varying conditions; it produced spores readily on gypsum blocks.   The reproduction was by budding, with frequent formation of joined bands. In rabbits intraperitoneal injections produced a fresh fibrinous peritonitis, with peritoneal nodules.   In some tissues the organism had an adventitious capsule.   In mice and rats brain lesions were produced, but no capsule formation occurred.   The pathological processes in general were those of degeneration and necrosis, with inflammatory reaction, which varied with the virulence of the culture.

The organism in this case is in a way a transition from the oidium type to the torula.   It resembles the oidium type in that the principal lesions it causes are cutaneous or subcutaneous, with internal lesions in the bones, lungs, kidneys, and spleen.   It has less attractive power for polynuclear leukocytes, sometimes has an adventitious capsule, and is known to produce brain lesions in animals, in these respects showing a resemblance to the torula group.   It differs from the torula group in not having a predilection for the nervous system in man, but in affecting especially the skin, and does not produce the gelatinous matrix.   Of course it is distinct culturally in its spore production.

The case of Hudelo, Duval, and Laederich, in which there were subcutaneous and cutaneous lesions, was exactly similar to Busse's, as regards all the characteristics of the lesions and parasites, as Buschke, who studied both, states.

In Curtis' case, where there were multiple subcutaneous tumors of a myxomatous appearance, with little proliferation of connective tissue, and some acute reaction, the organism usually did not occur in cells, measured 2 to 7μ in diameter, and had a clear capsule, with thin layers. In animals it grew in masses with an adventitious capsule, producing little reaction. No mention is made of the presence or absence of sporulation. Fermentation occurred. This case is anomalous. It does not exactly resemble either of the above groups; on the whole, it seems better to leave it unclassified until more observations are collected.

We defer a summary of the differential points of these three groups of true yeast, torula, and oidium infection, into which cases of systemic blastomycosis fall, until the cases to be presented in the paper have added further details.

In the remainder of the paper oidiomycosis will be used to designate the group of cutaneous and systemic diseases produced by organisms budding in tissue and producing mycelium in culture, especially noted near Chicago. The term blastomycosis confuses relationships and should be discarded.

III. TWO CASES OF TORULA INFECTION, WITH AUTOPSIES.

1. Case I.

A woman, aged 42 years, a native of Vermont, resident in Florida, entered the Surgical Service of the Peter Bent Brigham Hospital on Aug. 14, 1914, on account of headaches, failing vision, and dizziness of 3 months' duration, nausea and vomiting, diplopia, unsteady gait, difficulty in finding words, and disorientation.

Family History.—No record of definite nervous diseases, or of other significant troubles was obtained. Married for 18 years, with no children or miscarriages.

Past History.—Only minor illnesses. Always troubled with nervous headaches accompanied by lacrimation. Several attacks of

sore throat. Mild photophobia habitual. No history of cardio-respiratory, gastro-intestinal, genito-urinary, or skin diseases.

Present Illness.—About May 15 the patient began to have a severe headache, persistent and throbbing, at first frontal, but later occipital, accompanied for the first 2 weeks by a temperature ranging between 98° and 102°F. The headache did not stop; its most recent situation was in the back of the head and neck. Vomiting not preceded by nausea and not related to meals occurred twice, once 2 months before entrance and once a week before. After May 15 the patient's eyes ached and tired easily. Her vision, previously good, deteriorated until only the coarser type in a newspaper could be made out. Diplopia was definite in the 2 weeks before entrance. Dizziness and a staggering gait were present from the beginning of the illness. It was noticed early that the patient at times dropped words and found expression difficult. Faulty spelling and omissions occurred in her letters. Mental dulness began a month before admission, and 10 days before there was said to be complete disorientation for time, place, and person.

Previous Examination.—A choked disc; greater knee jerk on the right; negative Wassermann blood test; white count of 10,200; differential count of polymorphonuclears 58 per cent; small mononuclears 20 per cent; large mononuclears 12 per cent; eosinophils 10 per cent.

On entrance the following data were obtained. The patient was a fairly developed, rather poorly nourished woman, lying with closed eyes, tossing about restlessly, frequently throwing her hands up to the back of her neck and crying with pain in her neck, arms, and shoulders. She was dull, muttered unintelligible words, was completely disoriented for time and place, but apparently recognized her family.

Physical Examination.—Negative except for a blowing systolic murmur, loudest over the 2nd l.i.s., and also heard at the apex, and a white sticky pharyngeal exudate and a heavily coated tongue.

Cranial Nerve Examination.—A symmetrical head with marked suboccipital tenderness. Pressure over the neck and shoulders caused pain. Nerve I: the patient apparently did not recognize coffee. Nerve II: fundus o.u. disc distinctly obliterated; lamina cribrosa gone. Swelling of the cup estimated at 3 to 4 D. Partial and in places complete embedding of the large and tortuous veins. No

definite perivascular streaking. Arteries partially embedded. Macular region and peripheral fundus clear. Nerves III, IV, and VI: pupils vary in equality; both react to light; a tendency to exophthalmus. No ptosis. Apparently a slight weakness of the left external rectus. No definite nystagmus. Nerve V: negative. Nerve VII: question of some weakness of the right side of the face. Nerve VIII: slightly better hearing on the right. Nerve IX: definite dysarthria. Nerve X: pulse regular, very slight vomiting. Nerves XI and XII: negative.

Cerebrum.—Frontal: memory—some past memory; practically no recent memory. The patient knew where she was born and schooled, but did not know whether she had had breakfast or not. Orientation: completely disoriented for time and place, but not for person. Habits and disposition: marked change. The patient had become very disagreeable. Phases of negativism brought out during the examination were evidenced by poor coöperation, refusal of orders, etc. Temporal: a suggestive but not definite history of aphasia. None made out on examination, although a certain amount of dysarthria was present. No uncinate gyrus attacks or dreamy states. No hemianopsia on rough test. Parietal: negative for sensory and motor disturbances. Occipital: negative.

Cerebellum.—Romberg positive with eyes open, the patient tending to fall backward and more toward the left, even on a wide base. Gait: very uncertain, although the patient was able to walk, with staggering, especially to the left. Ataxia: negative. Tests: finger to nose test fairly well performed, right and left. Heel to knee test not coöperated in. Vertigo: some on standing. Diadokokinesia: fairly well performed.

Reflexes.—Superficial: epigastric, abdominal, and plantar not elicited. Deep: no biceps, triceps, patellar, tendo Achilles, ankle clonus, Babinski, Gordon, or Oppenheim on either side. Sphincters: not disturbed.

Positive Findings.—Objective: patient dull, muttering, disoriented for time and place, complaining of pain in the neck, arms, and shoulders; with suboccipital tenderness, questionable anosmia, marked choked disc, occasional inequality of pupils, possible weakness of left abducens, marked dysarthria, impaired memory, changed disposition and habits, positive Romberg, deviation in gait to the left, with uncertainty; and no obtainable superficial and deep reflexes, Kernig negative. Tongue and pharynx heavily coated.

Course of the Case.—On Aug. 15 a considerable resistance to flexion of the neck was noted. The left side of the face apparently moved less well than the right. There seemed to be great pain in the back and neck. No definite Kernig was found. Suboccipital tenderness and some fine nystagmiform twitches were discovered, but there was no outspoken nystagmus.

There was no marked extracranial dilatation of vessels, and no visible lack of parallelism of globes, although diplopia was complained of. The mental disturbances with rambling speech were striking features. The diagnosis was thought to depend upon internal hydrocephalus or general pressure.

Operation —On Aug. 15 a right subtemporal decompression was done. The dura showed no abnormality other than increased tension. On incising the dura the brain appeared tense, and exuded fluid freely. There was some excess of fluid in the subdural and subarachnoid spaces. No fluid was obtained on attempted ventricular puncture.

Aug. 18. X-ray examination of the head was negative. Eye examination showed a disc elevation of 1½ to 2 D with considerable perivascular streaking about the disc. Venous engorgement and tortuosity were still present to a moderate degree, and a few fine punctate hemorrhages in both discs, with partial embedding about the disc.

On Aug. 20 the left pupil was considerably wider than the right. On Aug. 21 Cheyne-Stokes respiration set in. The decompression wound was bulging and tense. There was marked carphology. The face was puffed more to the left, and the left lower side seemed weak. The head was constantly turned to the left, and the right leg was moved much more often than the left. The plantar reflex alone could be obtained. The temperature was 103°F.

On Aug. 27 examination of the chest showed dulness to percussion over the lower half of the left back with high pitched tubular breathing and many fine crackling râles. At the extreme base the note was flat and the signs were distant. A diagnosis of lobar pneumonia was made, with beginning pleurisy with effusion.

After a period of profound stupor, the patient began to respond imperfectly to questions. The left facial paralysis became more marked. The optic nerve head was 1½ to 2 D higher, and little punctate hemorrhages were visible in the left eye. The head was still held to the left and resisted straightening. The left pupil was still dilated The arm and leg on the right were never used spontaneously, but the right arm was drawn back on pin prick The right knee jerk was elicited for the first time. The hands moved aimlessly. There was a normal plantar reflex on the right.

The patient became rapidly worse, with cyanosis and dyspnea, and died on Aug. 27, at 7.30 p.m.

Clinical Pathology.—Urine examination: specific gravity 1,011 to 1,022; albumin, trace. Sugar 0. Sediment: bacteria, squamous cells, a few white cells, very rare granular casts.

Blood examination: white count, on Aug. 14, 11,000; Aug. 17, 16,200; Aug. 20, 34,300; Aug. 26, 25,200. Hemoglobin 90 per cent (Talquist).

Temperature course (rectal) on entrance 99.5°; on Aug. 15, 101.5°; on Aug. 17, 103.8°; then variations from 101.8° to 103.8° until 2 days before death when it was 102°.

Autopsy.—Permission for a complete autopsy was obtained and the examination made on Aug. 28, 13 hours post mortem. The body was put in a refrigerator immediately after death. The brain was hardened *in situ* by an injection with 10 per cent formalin through both carotid arteries.

Body.—The body is that of a slightly built, normally proportioned white woman, 165 cm. long. The skin is smooth, white, and free from edema or pigmentation. There are no discharges from external orifices, and no palpable glands. There is slight postmortem rigidity and lividity. The head shows a slightly bulging area, the site of a right subtemporal decompression operation. The pupils are equal and measure 4 mm. The chest is narrow and small. The breasts are slightly developed. The abdomen is level. The extremities show no abnormalities.

Primary Incision.—There is found to be a thin layer of abdominal fat and thin musculature.

Peritoneal Cavity.—The omentum is free from adhesions and contains a small amount of fat. There are about 20 cc. of clear, very slightly blood-tinged fluid in the pelvic cavity. The peritoneal surfaces are smooth, gray, and glistening, and there are no adhesions. The mesenteric and retroperitoneal lymph nodes are of normal size. The gastro-intestinal tract appears normal. The liver extends 4 cm. below the xyphoid and 3 cm. below the costal margin in the right mammary line. The diaphragm ascends to the fifth space on the right and the fourth space on the left.

Pericardial Cavity.—The pericardial sac contains about 10 cc. of clear yellow fluid; the surfaces are smooth and the heart lies entirely free within the cavity.

Heart.—Tricuspid valve 10.5 cm.; pulmonary valve 6.5 cm.; mitral valve 9 cm.; aortic valve 5.8 cm. Right ventricle 3 mm. thick; left ventricle 12.5 mm. thick. Weight 250 gm. The foramen ovale is closed. The endocardium covering the cavities is normal. Except for slight diffuse thickening of the edges of the tricuspid valve and slightly more focal thickening of the edges of the mitral valve with slight roughening of the endocardium, the valves appear normal. The

aorta shows a few small plaques of sclerosis, especially about the orifices of the coronary arteries. The coronary arteries are open throughout their extent. The heart muscle is pale and appears slightly yellowish, but is uniform throughout.

Right Lung.—The right lung is crepitant and slightly more firm than normally. The external surface shows a light deposit of carbon, and lividity at the base. The cut surface shows the carbon in a slightly moist, pinkish gray surface with distinct alveoli. At the tip of the apex is an area 0.5 cm. in diameter, apparently composed of fibrous tissue. The middle lobe has a slightly paler and moister external surface, but on section shows a reddish surface with scattered lighter areas apparently connected with the bronchi, exuding a thin white pus on pressure. The lower lobe is similar, but shows in addition slight congestion. The bronchi have a reddened mucosa with much purulent matter. The bronchial lymph nodes do not seem enlarged.

Left Lung.—The upper lobe is similar to the right upper lobe. The lower lobe is dark purple externally, and more solid than any of the other lobes. It is deep red and homogeneous on section. Pieces sink rapidly in water. The bronchi contain little pus, and there are no areas of induration about them. The bronchial lymph nodes are smooth and pale, with black streaks of carbon.

Liver.—Weight 1,205 gm. The external surface appears normal. Section shows greatly dilated and reddened central veins. The gall-bladder and bile ducts appear normal.

Kidneys.—Right weighs 120 gm.; left 145 gm. The right appears small and the capsule strips with some difficulty, leaving a finely granular surface in several places. The tissue cuts with slightly increased resistance. The vessels are congested. The glomeruli are visible. The left kidney is similar to the right except for a larger size, smoother surface, and less adherent capsule.

Spleen.—Weight 125 gm. The external surface is normal. On section it is pale with prominent Malpighian bodies.

Pancreas.—Appears normal.

Adrenals.—The medulla is soft,—almost liquefied.

Gastro-Intestinal Tract.—There is no sign of ulceration or inflammation.

Bladder.—Appears normal.

Genitalia.—The vagina appears normal. The external *os* is extremely small and leads into a small cavity into which the probe point of small enterotome scissors can hardly enter. Just at the level of the internal *os* the uterine wall contains a small nodule 1 cm. in diameter, whiter and harder than the rest of the uterine tissue. The left ovary is almost entirely converted into a sac containing bloody fluid. There is a slight amount of ovarian tissue surrounding the sac. The wall of the cyst has a smooth gray internal surface, and is about 1 mm. thick. The ovary is freely movable. The right ovary has a number of yellowish hard areas; on section one of them is circular, placed near the outer surface, and 4 mm. in diameter. The pelvic peritoneum covering the organs appears normal.

Aorta.—The aorta has a few sclerosed areas in the abdominal portion, especially

about the orifices of the branches, but they are slight, usually not measuring over 5 mm. in diameter, and little raised above the surrounding intima.

Lymph Nodes.—A calcified mediastinal lymph node is found, but the bronchial, mesenteric, and retroperitoneal nodes are free from signs of tuberculosis.

Head.—On removing the calvarium the dura is found to be free from adhesions to the skull except about the margins of the subtemporal decompression site. The dura is grayish white. The internal surface of the calvarium appears normal. When the dura is stripped back a number of not very strong white adhesions (more than the normal number) passing to the pia are found. The base of the skull appears normal.

Brain.—The brain is of normal size. The pia is definitely thickened, and more adherent than normally. On the right side of the brain in the temporal region, at the site of the decompression, is a hernial protrusion. The vessels and nerves at the base of the brain appear normal; there is no arteriosclerosis. On removing the meninges from the frontal lobes the convolutions appear slightly atrophied, with slight roughening of the surface by the presence of very small pits which are barely visible. There is some flattening of the convolutions over the whole brain. Asymmetry is evident, the right side being broader, especially in the temporal region.

On section the lateral ventricles are found to be dilated, the right more than the left. There seems to be no ependymal thickening, but the choroid plexus appears slightly thickened. The aqueduct of Sylvius is blocked in the posterior two-thirds by dense white tissue.

Serial sections of the whole brain, about 3 to 6 mm. thick, were made in a frontal plane. Frontal lobe: gray matter 3.5 to 5 mm. thick. Meninges in the depths of the sulci much thickened. The following lesions were found. In the left superior frontal gyrus, 2.3 cm. from the anterior tip of the lobe, is an area in the gray matter, bordering on the meninges, 2.5 mm. in diameter, filled with colorless gelatinous matter amid which are fine white dots and threads. Some of the threads are adherent to the edge, others pass across the middle. In the same convolution, 4 mm. further back, are two similar lesions in the gray matter at the border of the white matter; one 0.5 mm., the other 0.25 mm. in diameter, separated by a distance of 0.125 mm. In the same gyrus at the level of the tip of the caudate nucleus are

two similar lesions, 0.5 mm. from the upper surface of the gray matter, measuring 0.33 and 0.25 mm. The right superior frontal gyrus contains several similar lesions; one 0.5 mm. in diameter, just anterior to the caudate nucleus, another 0.25 mm. in diameter, 0.25 mm. further off. One at the tip of the caudate nucleus is 3 mm. in diameter. At the level of the anterior tip of the temporal lobes a lesion 0.75 mm. in diameter is found, 0.125 mm. from the upper surface of the cortex, and a similar lesion in the white matter 1 mm. below the gray matter. In the middle frontal gyrus is a lesion 0.75 mm. in diameter.

The right temporal lobe has an area 2 by 1.3 cm., extending mesially 1.5 cm. from the surface, and including the whole middle and part of the superior temporal convolutions, where there is loss of substance and granulation tissue (area of decompression). For 0.9 cm. back from this are minute spots of hemorrhage in the gray and white matter. Back of this decompression area, in the inferior frontal gyrus, just above the insula, is a lesion 1.6 by 1.5 cm., extending 1.6 cm. within the surface, of similar character. The internal capsule of this side is very soft.

Basal Ganglia.—The superior part of the putamen of the lenticular nucleus has many small lesions about 0.5 mm. in diameter, similar to those in the frontal lobe. Several coalesce into a streak 4 mm. long, Y-shaped.

Occipital Lobes.—Similar lesions in the precuneus, 4.8 cm. from the tip of the lobe. One is 1 mm. in diameter, and extends from the gray into the white matter; another is 2 by 1 mm.

Cerebellum.—In the right lobe, on the upper border, is a sharply circumscribed area 4 by 6 mm., of translucent rather mucoid appearance, surrounded by a white zone.

Corpus Callosum.—On the left is an area where the corpus callosum is swollen, softened, and apparently disintegrated. It is 7 mm. in diameter here as compared with 5 mm. on the other side. This is evidently the track of the needle used in the ventricular puncture.

The perivascular spaces throughout the brain appear slightly dilated, and often contain a small amount of gelatinous material.

Symmetry.—At the anterior end of the thalamus the greatest width—measured to the midtemporal convolution—is 7.7 cm. The left side at the same level is 6.1 cm. broad. The white matter on the right side appears much wider. The lateral ventricles have

abnormal shapes; the general alteration in angles is apparently due
to a combination of dilatation and drawing to the right which is the
result of the herniation.

The lesions in the right temporal lobe and the corpus callosum,
and the asymmetry, are evidently the result of operative procedures.

Anatomical Diagnoses.—Right subtemporal decompression, right
ventricular puncture. Internal hydrocephalus (slight). Occlusion
of the aqueduct of Sylvius. Subacute or chronic meningo-encepha-
litis of unknown origin, with multiple foci of infection in the cere-
bellum, cerebral cortex, and basal ganglia. Lobar pneumonia of the
left lower lobe. Left pleurisy with effusion. Slight bronchopneu-
monia of the left upper and right middle and lower lobes. Subacute
mitral endocarditis. Calcified mediastinal node. Passive conges-
tion of liver and spleen. Slight ascites. Slight chronic interstitial
nephritis. Leiomyoma of uterus. Stenosis of cervical canal. Cystic
left ovary. Postmortem softening of adrenals.

Microscopical Examination.

In general, a chronic leptomeningitis, consisting of the accumu-
lation of lymphoid, large phagocytic mononuclear, giant, and rarely
polymorphonuclear cells, with formation of connective tissue, especi-
ally about vessels, and with occasional caseous areas, is found over the
cerebrum, cerebellum, and spinal cord. Enormous numbers of spheri-
cal organisms, of varying size, with a definite wall, intra- and extra-
cellular, are present everywhere in the lesions. There are areas of
necrosis, but no circumscribed tubercles. Foci of infection of two
kinds are seen within the brain. One consists of a perivascular lesion
resembling the meningeal lesions. The other presents itself as a
small, spherical space in the cerebral tissue, filled with a non-staining
matter in which are embedded singly large spherical dark staining
organisms, which are occasionally seen budding. Few reactive cells
are seen within or around the lesion. The only reaction consists
of a very slight increase in glia tissue close to the lesion, and a moder-
ate number of large epithelioid cells and lymphocytic cells within the
lesion. The cerebellar lesion is of this second type. The more general
reactions of the brain consist of a marked cortical gliosis, and a diffuse
slight degeneration of ganglion cells and myelin sheaths.

Meningeal Lesions.—The meninges are thickened in varying degree, with connective tissue infiltrated with large phagocytic mononuclear, giant, lymphocytic, and plasma cells. The most extensive lesion is adjacent to the hippocampus; a somewhat less advanced lesion is in a section of the island of Reil. The lesions are more extensive in the sulci; in some regions of the cortex there is but little cell increase.

The advanced lesions show areas of necrosis or caseation, resembling that seen in tuberculosis. In detail, these areas include a caseous center, staining pink with eosin, in which indistinct remains of endothelial and lymphocytic cells, and organisms, can be seen. Often a moderate amount of fibrin, in fine threads, is present in the caseation, but is more apt to be seen at its border. Immediately surrounding the caseous material is a layer of elongated nuclei radially arranged, some of which have fibroglia fibrils along their periphery. Numerous large phagocytic endothelial cells and a few lymphocytes are present in this zone. Beyond is a thin band of connective tissue containing a few giant cells. In other places vessels may be seen surrounded by increased connective tissue, beyond which are caseous zones.

Between the areas of caseation the lesion consists of connective tissue of varying density, but on the whole reticular, in the meshes of which are included many plasma, giant, lymphoid, and large phagocytic mononuclear cells.

This chronic inflammatory tissue has many small spaces, entirely or partially filled with the organisms to be described later. These spaces for the most part are formed by the solution of the protoplasm of endothelioid or giant cells. Often in necrotic material a small space about 10 to 15μ in diameter is seen to contain an organism in the center. Giant cells are numerous and occur in two forms: one with eight to ten centrally located oval nuclei; the other with more spherical nuclei, peripherally located, and often entirely surrounding the central part of the cell. Organisms are at first peripheral in both forms, and occupy only the central portion after the outer portion has been destroyed or filled. The giant cells, as a rule, are not especially associated with nodular thickenings, but they occur diffusely. There are no areas of acute exudate. Polynuclear leukocytes are extremely rare.

Vascular Changes.—The most frequent vascular change in the

meninges is a proliferation of the adventitia of the vessels. Some-
times the endothelium is raised by a few mononuclear cells, or is
swollen, with a deposit of brownish pigment back of it.

Distribution.—Over the greater part of the cortex the meningitis
has not progressed to the point of necrosis or caseation, and corre-
sponds to the lesion just described as occurring between the areas of
caseation. Where there is the least amount of meningitis, the menin-
ges show slight thickening, a good deal of edema, and comparatively
slight cellular infiltration. A greater or less degree of meningitis is
present in all parts of the cortex.

Organisms.—Besides the organisms to be described later no others
could be found after search in sections stained with methylene blue
and eosin, the tubercle bacillus stain, and the Levaditi stain.

Reaction of the Cortex.—Throughout the cerebral cortex there is
great peripheral gliosis, varying somewhat in intensity. This, takes
the form of a dense cortical felt-work of fibers as much as 28μ thick
in the sulci, and 8 to 10μ on the convexity of the convolutions. In the
subcortical regions are large numbers of neuroglia spider cells, and
some increase in glia cells extends down to the ganglion cell layer.
The fibril formation is relatively more marked than the cellular
reaction, but the large cells in close relation to fibrils are very evident.
In the marginal gliosis no cells are seen.

Organisms in the Meningeal Lesions.—The size varies from 1.25
to 9μ. The large forms are spherical and have a wall 1μ thick; the
smaller forms, which occur most often, measure about 3.5μ in diam-
eter, and have a wall about 0.25μ thick; many are ovoid, the diam-
eters being 2.75 by 3.5μ, or 3.75 by 4.25μ. Measurements of some
of the smallest forms are 1.5 by 2μ, or 1.25 by 1.5μ; some appear to be
1μ in diameter. The wall is so thin in these very small forms that it
cannot be made out definitely.

The organisms are refractive and of a round or oval shape; the
larger ones have a wall appearing as a double line with a deeper
staining enclosed space; the smaller ones have thinner walls, so that
those about 3μ in diameter have a wall appearing as a single line of
definite thickness. As stated above it is difficult to make out the
presence or absence of a wall in the very smallest forms, but the reg-
ularity of shape and the refractility suggest that there is a defi-

nite membrane about the protoplasm. Sometimes the organisms stand out prominently; at other times they are difficult to see without special stains. With hematoxylin the inner line of the wall stains as a definite, sharply defined line, slightly thicker than the extremely thin line outlining the outer surface. Cresyl blue gives a similar staining reaction. With both, as well as with methylene blue, the wall stains diffusely, so that the double contoured part appears darker than the central part. Partly degenerated organisms lose their staining properties. Mallory's aniline blue connective tissue stain colors the walls of the organisms a faint blue, and they stand out prominently in contrast to the red tinge of the surrounding tissue. Levaditi's stain colors them a yellowish brown, giving a strong contrast to the light staining tissues. The very small organisms do not show evident coloration of the wall with any staining reagent tried; consequently their presence might easily escape notice; but this absence of coloration makes it easy to distinguish them from nuclear detritus; while their refractility makes a less easy distinction possible from products of degeneration. A stain with iodine results in slight brown coloration of the outer part of the wall of the largest organisms; in the smaller ones no staining can be seen.

Interior of Organisms.—Usually eccentrically placed, is a small particle, about one-sixth the diameter of the organism, roughly spherical, which takes nearly all stains slightly,—cresyl blue, Marchi, methylene blue, and Scharlach R. It is present especially in the smaller organisms. In the largest ones it is almost invariably absent; and it is often absent in organisms of all sizes. In frozen sections stained with cresyl blue the cell interior is best made out,—and then it is found that besides the material just described, there are varying numbers of particles about one-half its size. The masses are sharply outlined, and vary in size and number without relation to the size or budding of the organism. In formalin-fixed material teased out and mounted in glycerine or in salt solution plus a drop of 10 per cent sodium hydroxide, these masses appear more refractive than the organism, but less refractive than fat; they have the same refractility as the droplets usually present in fungi. The organisms do not show a clear zone between the cell wall and the cell protoplasm, as is usual in the organisms of blastomycosis. The

study of the cell interior is unsatisfactory in the meningeal lesions
on account of the age of the lesions and the degeneration of most of
the organisms.

Variations from the Usual Forms.—Occasional organisms are irregular
in outline, appearing partially collapsed, or are broken in places, or are
open showing a cup-like interior.   These broken forms, except in their
size, resemble red blood corpuscles.   They also differ in often having a
jagged or pointed edge, and in having different staining properties.

Reproduction.—The organisms occur in enormous numbers in the
meninges, but evidence of a method of reproduction is at first difficult
to find; probably because the lesion is a very old one and not pro-
gressing.   The empty capsules suggested endosporulation, but no
evidence of such a process could be found.   Budding was more strongly
suggested by the frequent occurrence in apposition of a large form and
a small form.   Many definite buds were found, the protoplasm of
the larger cell flowing out, then becoming constricted to form a new
cell one-third to one-half the diameter of the original one.   In one
case a cell 3.75 by 4.25μ was producing a bud 1.5μ in diameter,
which was partially separated by constriction.   Budding occurred
most frequently in the medium sized forms; it was made out definitely,
however, in the largest and the smallest forms.   The buds have thinner
walls than the parent organisms; some have no visible wall.

Occurrence of Organisms.—The greater part of the organisms occurs
in giant cells.   Sometimes one-half of a giant cell is represented by a
space in which are four or five organisms; one arrangement is a space
containing one organism about 3 to 4μ in diameter, and three to four
organisms 1.5 to 2.5μ in diameter.   The organisms lie in the space
separated from each other, unless they are budding.   In other cases the
organisms pack the giant cells full of spherules 3 to 4μ in diameter,
the protoplasm and nuclei of the giant cell having entirely disappeared.
Sometimes the bodies occur diffusely through the outer portions of
the giant cell, with very slight spaces, or no spaces, about them.
These organisms usually show no central masses.   The organisms
are often found in phagocytic mononuclear cells, having the same
relation to the cell as in the giant cells.   Many organisms occur
extracellularly.   These usually have a more or less spherical space
around them about two to two and a half times their diameter,

especially when the organisms are large or when they occur in necrotic material. The spacing of the organisms in necrotic tissue gives a honeycombed appearance.

The larger organisms often have fine threads connecting them, which stain easily with methylene blue. The threads are a little larger near the organisms, taper slightly to a place midway between them, are a little irregular in outline, apparently are attached to the cell wall, and cannot be made out to have a wall themselves. Occasionally they are apparently formed by the separation of budding cells, and on the whole do not appear at all like mycelium. They suggest some mucoid or gelatinous substance which is drawn out into threads as reproduction and separation take place, or is shriveled into filaments by the action of a fixative; in short, they represent some extracellular substance about the organisms. These filaments are more prominent in the intracerebral lesions than in the meninges.

Relation of the Organisms to the Cells.—The phagocytic giant cells show all stages of degeneration. There may be spaces like those just described, probably resulting from a solution of the substance of the cell; the nuclei become pyknotic, the remainder of the protoplasm becomes finely vacuolated and stains faintly; finally the nuclei disappear; then the protoplasm vanishes, and a space which is occupied by a few organisms or packed full of them remains, bordered by the old edge of the cell. When the organisms occur without spaces about them the giant cells do not show such degeneration; it is probable that in this case the giant cell has taken up dead organisms which have not been able to destroy it.

Intracerebral Lesions.—In the cerebral cortex, in the cerebellum, and in the basal ganglia are lesions peculiar in the mode of extension and in the reaction.

The Cerebellar Lesion.—This lesion measures 0.45 cm. in diameter and consists essentially of a solution of the cerebellar cortex and a filling up of the space by a colony of organisms, each separately placed in a gelatinous matrix. Strands of tissue are seen crossing the cavity, consisting of remains of the granular and molecular layers, in apparently good condition. No layer seems to be more susceptible to the destructive influence than another, and the almost spherical outline of the lesion shows that the periphery is being extended uni-

formly without regard to the tissue. The extension is plainly by solution of the tissue, and not by the pressure of the organized mass. Small masses of organisms, or even individual organisms, can be seen sinking into the molecular or granular layers. Numbers of hyaline remnants of cells, some of which were apparently large phagocytic mononuclear cells, others lymphocytes, float in the cavity, and in one place form a layer at the periphery. The small organisms often pack these cells. No polymorphonuclear, plasma, or red blood cells, no newly formed connective tissue, and no bacteria can be found within the cavity.

Organisms in the Cerebellar Lesion.—The usual size is from 9 to 13μ. Smaller ones 6μ in diameter are frequent; occasional organisms are 3.5μ in diameter. Near the periphery of the lesion organisms 1.5 to 2μ occur rarely.

The organisms are spherical, and thick walled. The wall stains a deep blue with methylene blue. Perhaps on account of the thickness of the cell wall, no differentiation of the cell interior can be made out. The organisms are homogeneous throughout, with a certain clearness quite different from the solidity of corpora amylacea, and the frequent occurrence of broken forms shows them to be hollow spheres with thick walls. Often the outline of the organism is uneven, as if there had been crushing, or partial collapse from the process of dehydration. The smaller forms appear like the organisms in the meninges. Threads connect the organisms as in the meninges, but they are thicker and more definite in this lesion. With a cresyl blue stain it is seen that the lesion is nearly filled with a substance staining faintly which surrounds the organisms. This is irregular in outline, very variable in extent, and resembles a mucoid or gelatinous material which has been formed as a result of the activity of the organisms and has constricted during the processes of preparation, so that it adheres to and surrounds the solid materials in the cavity. The threads seen in the methylene blue stain are thickenings in this material, formed by the drawing apart of the organisms or the shrinkage of the material. With the cresyl blue stain radiating thickenings often project from an organism in great numbers, giving it a star-like appearance. Rusk has described similar forms and they resemble the gelatinous deposit described by Klein, Cohn, Busse,

and Nichols in their experiments with torulæ, and seen by us in our experimental lesions in animals. It results in holding the organisms in their peculiar separate placing. Budding forms are rare in this lesion. Sometimes there is an appearance as of successive buds, the budding cell itself budding before it is separated. No appearances at all suggestive of endospore formation are seen. These large forms with thick walls and few evidences of reproduction seem like involution forms or like resting cells. They suggest the large cells found in old cultures of torulæ. The conclusion is evident that the lack of reaction is not to be explained by a postmortem invasion of the organisms; these large thick walled forms with little evidence of activity and the presence of the desquamated mononuclear cells indicate the end-stage of an old process.

Reactions of the Cerebellar Tissue to the Lesion.—On account of the relations of the meninges in the cerebellum it is difficult to say what reaction is due to the meningeal lesion and what to the internal lesion. There is a slight but definite increase in the glia tissue of the cerebellum near the lesion, most marked where the infected meninges are close; and since it is also plainly visible in the strands of molecular layer floating in the lesion, and indeed is more marked here than beyond the periphery, it is probable that there is a slight reaction of the glia to the internal lesion.

Degenerations in the Cerebellum.—On one side of the lesion the Purkinje cells appear slightly shrunken and elongated, the nucleus is poorly marked, the Nissl bodies are not clearly cut, and, toward the periphery of the cell, the cell protoplasm takes an abnormally deep blue with methylene blue and has a somewhat cloudy appearance. This change is more marked near the lesion than at a distance from it; but around a considerable part of the lesion no change is demonstrable.

Other Lesions within the Brain.—Lesions similar to the cerebellar lesion in all respects except size are found in sections of the basal ganglia. There is the same slight invasion with the large phagocytic mononuclear cells, the same slight reaction of the glia cells, the same absence of all acute exudate even in the smallest lesions, the same method of extension by solution of brain tissue with the formation of new lesions, and the same spacing and form of organisms. The

differences are that the organisms are, as a rule, smaller than in the cerebellar lesion; the average size is 7 to 8μ instead of 10 to 11μ; the largest is 9μ instead of 13μ; a good many are 4.5 to 5μ, and they occur as small as 2μ. Many of the medium sized or smaller organisms are seen to contain a protoplasm staining dark red, irregularly granular, without definite nucleus. The protoplasm fills the cell. Budding organisms are more frequent here than in the cerebellum.

The cerebral tissue adjacent to the lesion shows little or no change. The pointed projections between the organisms show how these sink individually into the tissue. Often no gliosis can be made out; at other times there is a slight increase in glia cells, more rarely in glia fibrils.

Stages occur from these comparatively non-reactive lesions to perivascular lesions similar to the meningeal lesion. There is more and more cellular infiltration and increase of connective tissue, and less evidence of the peculiar extension by solution of tissue, the organisms becoming more and more intracellular and smaller in size. Often the perivascular spaces become considerably dilated.

Another type of cortical lesion consists of the thrombosis of a vessel with many organisms in the thrombus, with dilatation of the vessel wall, and extension through it, forming a lesion of the first or second type. It is not possible to say whether all the lesions in the cortex arise in this way. It would not be hard for the minute forms of the organisms to pass through the vessel walls, and in fact evidence of this was seen in three cases, although on account of the hyaline character of the forms definite conclusions are impossible.

A fourth, and fairly frequent type of cortical lesion consists of the thrombosis of a vessel, with fibrin and blood cells in the lumen and a surrounding exudation of lymphoid, large mononuclear, and plasma cells, with formation occasionally of connective tissue. In these lesions organisms were never found. It is possible that they are the result of the occlusion of the vessel further on by the organisms, or the organisms may have been destroyed.

Distribution of the Brain Lesions.—As in the gross examination, the greatest numbers of intracranial lesions are in the frontal and olfactory lobes, and in the basal ganglia.

Degenerations.—Many of the small pyramidal cells, and a less number of the large ones, show evidence of slight degeneration;

the cells stain faintly, the Nissl bodies are not made out, often the nucleus is faint or invisible or shrunken and pyknotic, irregular in shape or elongated. The cell body at times appears shrunken, the cytoplasm irregularly stained. In the large pyramidal cells there is apt to be a clear zone around the nucleus, with irregular staining of the periphery. The polymorphous cells appear normal. In the medulla the ganglion cells appear more normal, but here the Nissl bodies are usually not made out and the cell body is occasionally shrunken. The ganglion cell changes are more marked in the frontal region. Here especially ganglion cells are often seen with two to four trabant cells about them. Marchi stains show a slight diffuse deposit of osmic acid in the myelin sheaths throughout the brain.

Miscellaneous Lesions.—The third ventricle shows lesions in the choroid plexus of the velum interpositum similar to those in the meninges, except for the absence of necrotic areas, and a larger proportion of phagocytic endothelioid cells. One foramen of Munro is partially occluded with a perivascular lesion. Sections of the lateral ventricles show similar conditions in the choroid plexus.

Distribution of Basal Ganglia Lesions.—In the gray matter of the median thalamic nucleus are many small lesions, the largest about 0.1 mm. in diameter, for the most part of the non-reactive type. In one section 5 by 8 mm. in size, twelve small lesions are found. The vessel sheath spaces are often greatly dilated and contain plasma cells or lymphocytes.

Peripheral Nerves.—Organisms are packed beneath the nerve sheath of the eighth cranial nerve and are in the capillaries of the same cranial nerve.

Aqueduct of Sylvius.—Sections of the occluded aqueduct show a mass of. chronic inflammatory tissue with much vessel formation, similar in general character to the meningeal thickening, and containing many organisms, which completely fills the lumen. The wall of the aqueduct is thickened with glia tissue.

Ventricles.—About the ventricles there is a dense gliosis.

Spinal Cord.—There is slight meningitis of the same character as in the cerebral meninges.

Lungs.—In most sections there is a typical acute bronchopneumonia in which no torula organisms can be found. Section of the left lower

lobe shows a pneumonia with alveoli filled with polymorphonuclear leukocytes, red blood cells, lymphocytes, endothelioid cells, and fibrin.

Liver.—No lesions. A slight thickening of the portal connective tissue.

Spleen.—There are many nodules the size of miliary tubercles, composed of epithelioid cells with poorly staining nuclei and a few small giant cells. Although there is no central caseation the nodules closely resemble the more chronic type of miliary tubercles. There is sometimes some central necrosis. The nodules are scattered throughout the tissue without reference to the follicles. No organisms of any kind can be found in these nodules. The arteries of the follicles show hyaline degeneration, and there is slight hyaline in the reticulum.

Kidneys.—A chronic lesion, about 2 mm. in diameter, composed of a diffuse interstitial infiltration with lymphoid and plasma cells, without caseation, occurs near the pyramids. In giant cells and free are organisms which stain faintly and are evidently partially destroyed. Other lesions consist of small accumulations of lymphoid and plasma cells, without visible organisms, in the renal cortex. Degeneration of near-by tubules is evident.

Heart.—No lesion. Histological changes probably postmortem in origin. Sections of the mitral valve show organized vegetations and cicatricial connective tissue.

Adrenals.—No lesions. Postmortem changes only.

Bronchial Lymph Node.—A small amount of carbon deposit, a diffuse increase in connective tissue, a somewhat thickened capsule, and hypoplastic germinal centers.

Ovary.—Some areas of hyaline connective tissue occur and there are collections of large pale cells.

A. Discussion of Case I.

Explanation of the Clinical History.—Evidently the internal hydrocephalus, to be explained by the stoppage of the aqueduct of Sylvius by chronic inflammatory tissue, together with the chronic meningitis, is sufficient to explain the general pressure symptoms of long duration. The effect of the long continued pressure explains in part the cerebral symptoms; the more localizing symptoms in the frontal lobes, basal

ganglia, cerebellum, and cranial nerves are to be further explained by the lesions in these regions found at autopsy. Lesions such as these, with their chronic course and variety of situation, are evidently capable of simulating brain tumors with definite localizations so closely that from the symptoms alone a differential diagnosis would be impossible.

That the organisms are the causal factor in the lesions is proved by the peculiar character of the lesions, by the occurrence of the organisms in all the lesions in enormous numbers, by their occurrence nowhere else, and by the absence of any other demonstrable agent. It is unfortunate that cultures could not be obtained and Koch's laws fulfilled, but experimental lesions have been produced by us in animals by torula organisms, which are so exactly like those of this parasite in morphology and in reaction that there can be no doubt that the organism found in this brain is a torula. The morphology of the parasite with the clear homogeneous appearance of the large ones, the small number of differentiated droplets in the small ones, the tendency to multiply in and dissolve cells, to produce solution of brain tissue with only a slight chronic reaction; the formation of a wide clear zone about the outer wall, demonstrated by special stains to be caused by a gelatinous deposit in the lesion which keeps the organisms far apart and becomes converted into threads connecting the organisms, and the good staining properties of the cell wall, all differentiate this organism distinctly from the oidium, which has a finely granular content separated from the wall by a clear zone, a faintly staining or non-staining cell wall, slight or no production of gelatinous material so that organisms lie close together, and a tendency to attract polynuclear cells and cause a proliferation of fibroblasts. This organism is distinct from the coccidioidal organism in the smaller size, the presence of budding, the absence of endosporulation, and in the failure to attract leukocytes. The case is one of torula infection and is closely similar clinically and pathologically to the cases collected from the literature.

How can the absence of reaction in the intracerebral lesions of long duration be explained? There is a reaction in the meninges, so that the lack of reaction in the brain cannot be due to the peculiar nature of the organism. In morphology the parasite is old and slowly grow-

ing, so that the lesions could not possibly be explained by an invasion near the time of death. Something prevents the reaction of the cerebral tissues, and the most probable substance which could do this is the gelatinous substance peculiar to these lesions. Probably this gelatinous deposit greatly hinders all attempts at destruction of the parasite by the host. The general gliosis cannot be ascribed to the action of the organisms, but is to be explained, for the most part at any rate, as the result of the long continued pressure due to the internal hydrocephalus. The resulting interference with circulation may also explain the degenerations found.

The solution of tissue confined to the immediate neighborhood of the organisms suggests that the poisonous products are very slightly diffusible or are neutralized immediately.

Whether the intracerebral lesions arose from the meningeal lesion by the carrying of organisms along the perivascular sheaths, whether the reverse occurred, or whether both occurred independently, it is impossible to say. No direct connection between a meningeal lesion and an intracerebral lesion was ever found, except in the case of the cerebellar lesion. We can only say that probably in many cases the infection extended by way of the perivascular spaces.

The resemblance of the meningeal lesions to tuberculosis is interesting. The chief differences between these lesions and those of tuberculosis are the absence of perivascular infiltration, the diffuse distribution of giant cells without reference to nodule formations, the large number of plasma cells in the tissue, and the occurrence of spaces in the giant cells and tissues filled completely or partially with organisms.

2. Case II.

The second case was that of a man, 39 years old, a teacher living in Boston, who entered the Surgical Service of the Peter Bent Brigham Hospital on Nov. 8, 1914. The history was complicated, and the diagnosis had been uncertain. The main symptoms were cerebral, and included a right hemiplegia, dizziness, and drowsiness, and a complex referable to increased intracranial pressure. The clinical record follows.

Family History.—Negative.

Past History.—A severe attack of scarlet fever at 3 years of age. Frequent colds with cough, but no chronic bronchitis, or chronic cough with raising of sputum. No history of hemoptysis. Syphilis and gonorrhea were denied. The highest weight was 180 pounds, 14 years before entrance; at entrance it was 170 (average weight). There had been no cardiac or gastro-intestinal symptoms. Nocturia occurred one or two times. There had been no edema. There was an error of refraction, for which glasses had been prescribed 7 years before entrance, without subsequent correction. The secondary sexual characteristics largely failed to develop. In March, 1914, an acute middle ear was opened and drained with good result.

Present Illness.—In the latter part of September, 7 weeks before entrance, pain in the right ear came on. The ear was opened and drained and a small amount of pus was obtained. The pain was relieved and for a week the patient felt well. 6 weeks before entrance a frontal headache began, with a temperature of 102°, and the patient went to bed. 3 days later, on awaking, he found that he had a right hemiplegia, affecting the arm most of all, then the face, and to a less degree the leg. The hemiplegia gradually cleared up, except for a residual weakness of the right arm. During this same period of 6 weeks drowsiness was a marked symptom, the patient sleeping most of the time during the day. There was no loss of vision.

The patient was at the Massachusetts General Hospital under the observation of Dr. F. T. Lord, from Sept. 21 until his admission to the Peter Bent Brigham Hospital. During his stay there two spinal punctures were made. The first one showed 57 cells (lymphocytes) per c. mm. The Wassermann test was negative, the Noguchi positive, the gold chloride test positive for tumor or tuberculosis. The second puncture fluid had 21 cells per c. mm. Here again the Noguchi test was positive and the Wassermann negative, but the gold chloride test was positive for syphilis. A Wassermann test on the blood serum was again negative. Cultures of the spinal fluid were made on blood serum, and no growth was observed within 48 hours. A guinea pig was inoculated with the fluid, and later killed, with negative results.

The eye grounds were negative on several examinations in September and the early part of October.

Salvarsan was given twice, first on Oct. 16, 0.1 gm., then on Oct. 23, 0.3 gm. Previous to the salvarsan, mercury inunctions were used for about 3 weeks. The night before the second administration of salvarsan the patient complained of headache. After the inoculation, fever, occipital and bitemporal headache, dizziness, and vomiting came on. The temperature soon went down, but the headache and dizziness persisted until entrance to the Peter Bent Brigham Hospital, and vomiting occurred three times without nausea. On Oct. 26 examination of the eye grounds showed an early choked disc without measurable swelling.

An x-ray of the chest showed a diffuse shadow at the roots of both lungs. The tibia had some thickening of the bone.

Physical Examination at Entrance to the Peter Bent Brigham Hospital.—A right-handed man lying in bed with his eyes closed, apparently from photophobia, complaining of pain in the eyes. There was evident weakness of the right face and arm, and some puffiness under the eyes. The veins of the upper lids were dilated and the globes were prominent. The head had no areas of tenderness or change in percussion note.

Cranial Nerve Examination.—Nerve I: normal. Nerve II: the ophthalmoscope showed in the right eye obscured margins on the temporal side, new tissue in the cup, and a disc raised 2 D. The left eye had a more advanced choked disc. There was a height of 3 D, with embedded vessels, and one punctate hemorrhage. The surrounding retina showed considerable edema; the visual fields were practically normal. Nerves III, IV, and VI: the pupils were irregular, small, and reacted sluggishly, especially on the left. No von Graefe's sign, no nystagmus. Movements of eyeballs not restricted. Slight exophthalmus, no diplopia. Nerve V: no subjective disturbance. Masseters and temporals equally strong. Pain, touch, and temperature well and equally recognized. Nerve VII: the right corner of the mouth was weak, and the right nasolabial fold smoothed out. The eyes closed and the forehead wrinkled equally well on both sides. Nerve VIII: a watch could be heard at 3 feet on each side. History of right hemiplegia. Air conduction was better than bone conduction on both sides. Galton whistle was well heard. Weber's test was heard, but not referred to either side. Nerve IX: no history of difficulty in swallowing and speaking. Nerve X: pulse regular, 64 to the minute. History of vomiting three times. Palate symmetrical. No vocal cord disturbance. Nerve XI: sternocleidomastoid and trapezius equally strong on both sides. Nerve XII: tongue protruded in median line. Movements normal; no apraxia. Except for the impairment

of motor power on the right, there were no symptoms referable to the frontal, temporal, parietal, or occipital regions. Dynamometer test: right 10 kilos; left 70 kilos. All the muscle groups of the right arm were markedly weak. The right leg was not greatly impaired. There were no sensory abnormalities; no astereognosis. Cerebellar examination showed dizziness; no nystagmus; inability to stand on account of weakness, but no history of unsteadiness; impossible diadokokinesia on the right, normal on the left; finger to finger and heel to shin tests fairly well performed, allowing for weakness of the right arm and leg.

Reflexes.—The abdominal and plantar were absent on the right. The epigastric was not elicited. The cremasteric, plantar, corneal, triceps, and biceps were present on both sides. The knee jerks were active to exaggeration, especially on the left. The Achilles jerks were active on both sides. Clonus was absent on the left, poorly sustained on the right. Babinski and Oppenheim were absent.

Examination of the right ear showed a little clotted blood about the external meatus, a dark blackish green membrane with no light reflex, and a small scar at the center. The left drumhead was bulging a little and reddened around its margin, with dim reflex. X-ray of the head was negative. The urine had a specific gravity of 1,750; albumin 0; negative sediment. The white count was 9,100; hemoglobin 75 per cent (Talquist). The temperature was normal. Wassermann reaction on the blood serum was negative.

Positive Findings, Subjective.—Pain in the right ear, two attacks, in March and September, 1914. Impaired vision of 2 weeks' duration. Headache and fever for a few days 6 weeks ago. Right hemiplegia, beginning 6 weeks ago and clearing up partially by the time of entrance. Dizziness of 2 weeks' duration. Headache of 2 weeks' duration, located in eyes. Vomiting three times in the last 2 weeks, following the administration of salvarsan. Drowsiness of 6 weeks' duration.

Positive Findings, Objective.—Weakness of the right face, arm, and leg. Exophthalmos. Dilated venules of eyelids. Choked disc, more marked in the left eye. Sluggish pupils. Exaggerated deep reflexes, especially on the right.

Wassermann reaction (blood serum) negative.

Progress of Case.—On Nov. 13 a left subtemporal decompression operation was done to relieve the increased intracranial pressure. The dura was found to be quite tense, and there was considerable fluid. The arachnoid and brain surface appeared normal.

The wound healed quickly and well. At first the headache was less, but in a few days it returned and frontal symptoms, such as dis-

orientation and loss of memory, with failure to recognize his family, became more and more evident. Suboccipital headache was then complained of. Drowsiness continued. By Nov. 21 the suboccipital headaches had become very severe, and disorientation and hallucinations were marked. Incontinence of feces and urine at night with great restlessness were noted by Nov. 25. The eye grounds showed no further changes. On Nov. 27 the headache seemed to be mostly frontal. Examination on this date showed a deviation of the jaw to the left. The disc swelling was 4 D, and the decompression very tense. Vomiting occurred occasionally. There was an inspiratory hiccough, and deviation of the tongue to the right. There were wrist clonus and ankle clonus on the right. On Nov. 30 some apraxia was noted. On Dec. 1 spinal puncture gave 15 cc. of fluid, not under increased pressure. There was 1 cell per c. mm. (lymphocyte), and about 60 red blood corpuscles. On the same day eye examination revealed a swelling of the discs of 4 D with exudate and edema spreading far over the retina. On the left were many flame-shaped hemorrhages. The tentative diagnosis at this time included temporal lobe abscess, unilateral hydrocephalus, rapidly growing glioma, or vascular lesions.

Operation.—On Dec. 4 an osteoplastic exploration was made. The brain was incised to a depth of 3 cm., in the posterior part of the field, and a needle inserted through this opening, through which about 150 cc. of cerebrospinal fluid were obtained. There was definite improvement following operation, but for only a brief period. Pulse and respiration became irregular. A ventricular puncture was made through the former opening, and 175 cc. of slightly turbid fluid were withdrawn, in which 1 cell was found per c. mm. A Wassermann reaction on the spinal fluid on Dec. 4 was negative. On Dec. 11, 200 cc. of ventricular fluid were obtained, and a Wassermann reaction on it proved negative. Following the puncture came relief of the suboccipital headache.

Ventricular punctures were made, with the following results:

Dec. 17. 150 cc. of yellowish fluid, 60 red cells.
 "   22. 30 " " spinal   "   4 "   "
         90 " " ventricular " 18 "   "
 "   27. 118 " "   "   " 78 "   "
Jan. 2. 130 " "   "   " 84 "   "   3 white cells.

Examination of the fluid gave: albumin, 0.016 to 0.12; sugar, 0.0786 to 0.0972.

It is of interest that on Dec. 22 the spinal fluid soon ceased flowing, while the ventricle, punctured at the same time, continued to give forth fluid. This seemed to prove the absence of a free communication between the ventricle and the spinal subarachnoid space.

On Dec. 7 Cheyne-Stokes respirations were noted at times. The patient became more stupid and refused nourishment except milk. The pulse became irregular, there being a pause every 6 to 10 beats. On Dec. 8 the irregularity of pulse and respirations entirely disappeared, but returned on Dec. 11. On Dec. 12 paralysis of the right side was practically complete, and there was marked aphasia. On Dec. 28 there was difficulty in breathing, with rapid, noisy respirations. On examination of the lungs coarse râles were heard over the trachea.

After the last puncture there was the usual temporary relief of symptoms, and during the evening the patient seemed brighter than usual. The pulse was good, the respiration regular. The patient died during the early morning.

Autopsy.—The autopsy was performed 5 hours post mortem. The brain was hardened *in situ* by an injection of 10 per cent formalin through the carotid arteries.

Externally the body is that of a moderately well nourished man and shows no abnormalities other than a herniation of the brain in the left parietal region. No lymph nodes are palpable. There is no pigmentation of the skin. No scars are visible. The testes are large and firm.

The peritoneal cavity appears normal.

In each pleural cavity, at the apices of the lungs, are found a few string-like adhesions. The cavities are free from fluid.

The pericardial cavity appears normal.

Heart.—Weight 350 gm. The foramen ovale is patent to a diameter of about 4 mm., but the valve-like action of the folds of endocardium about it must have made the opening functionally unimportant. The valve segments all appear normal except for a very slight fibrous thickening of the edges of the mitral valve. On the aorta are several spots of sclerosis about 2 mm. in diameter. The coronary arteries show a slight sclerosis, but the lumen is fully patent.

Lungs.—The pleural surface of the apex of the right lung is deeply puckered with folds from 1 to 1.5 cm. deep. The upper lobe is shrunken and has a large area of consolidation, which cuts like hard fibrous tissue, and is composed of small,

firm grayish white nodules varying in size up to 2 mm., not circumscribed, and set in a hard slate-gray tissue. The mass as a whole is irregular, with nodular extensions into the surrounding tissue. It is dry and anemic looking. The consolidation extends to the pleural surface and is surrounded by fibrous masses extending into the lung. There is no calcification, nothing even suggesting caseation, and no cavity formation. The bronchi pass into the fibrous area with dilatation and are lost in it. In the lung elsewhere are scattered small miliary nodules resembling those in the consolidated area, and on the pleural surface are a number of small flat nodules, not elevated, extending in pointed processes into the lung beneath. Some of these are in small masses.

The left lung shows a similar condition, but the consolidation is more extensive, and occupies most of the upper lobe, even to the root of the lung. The puckering of the surface is very marked.

The bronchial lymph nodes are enlarged, pigmented, indurated, but neither caseated nor calcified. The mediastinal nodes are similar to the bronchial, showing on section yellowish white areas.

Liver.—Weight 1,920 gm. The external surface and cut section appear normal. The bile passages are open and appear normal.

Spleen.—Weight 280 gm. When cut it is found to be soft, scraping off easily with the knife. The surface is deep red, with prominent Malpighian bodies.

Pancreas.—Appears normal.

Gastro-Intestinal Tract.—Appears normal.

Kidneys.—Weight of each 200 gm. The capsule strips easily, leaving a smooth deep red surface. On section deep congestion is seen, with prominent markings.

Adrenals.—Slight postmortem degeneration.

Bladder, Prostate, Seminal Vesicles.—Appear normal.

Aorta.—Slight diffuse sclerosis of the abdominal portion.

Head.—There is considerable adhesion about the site of the bone-flap operation.

The calvarium, on removal, appears normal, as does the external surface of the dura. When the removal of the brain is attempted, it is found that there are very strong adhesions in both cerebellar fossæ, which prevent the removal of the cerebellum without tearing it. There is no exudate here, however, or evidence of acute inflammation. A piece of the dura with adherent cerebellum is put into Zenker's fluid at once. The middle ears are opened. The right appears normal, the left shows a slight amount of mucoid material.

Brain.—There is a large bulging mass on the left side at the site of the decompression operation. The convolutions are flattened. The dura, except in the immediate vicinity of the wound, is not

adherent. The pia is slightly thickened and cloudy, but hardly to a noticeable extent. On the upper surface of the cerebellum, particularly on the right side, but to a certain extent elsewhere, there is a very definite thickening of the pia arachnoid, which has stripped smoothly from the dura, but is firmly adherent to the cerebellum. The same thickening of the pia arachnoid with formation of adhesions is found posteriorly, more marked on the right.

The brain is then cut into parallel frontal sections from 3 to 6 mm. thick.

Section through the frontal lobe shows dilated ventricles and normal cortex. In a section through the anterior commissure there is found on the left side, just above the anterior commissure, in the lower extremity of the internal capsule, a lesion 4 by 2 by 3 mm. anteroposteriorly, yellow, soft, with loss of brain substance.

5 mm. further posteriorly, at the level of the amygdaloid nucleus and the anterior tip of the caudate nucleus, is a lesion 6 by 3 mm., extending 6 mm. posteriorly, consisting of an area of induration with a yellow soft spot in the center, situated in the globus pallidus and lower part of the internal capsule on the left.

A section through the head of the caudate nucleus shows greatly dilated ventricles. The dilatation is equal on both sides. The horizontal diameter of the ventricles is 2.2 cm., the greatest vertical diameter (measured diagonally) 3.3 cm.

At the level of the tips of the anterior horns of the ventricles the cortex of the superior frontal convolution is 2.5 to 3.5 mm. thick, of the midfrontal 2.5 mm., of the inferior frontal 3 to 3.5 mm., of the uncinate gyrus 1.5 to 2 mm.

In the lenticular nucleus on the right, and extending very slightly into the internal capsule, is an indurated pale area 4 by 2.5 mm. made up of several spherical nodules 1 to 2 mm. in diameter. It extends 9 to 10 mm. anteroposteriorly.

The third ventricle, the foramen of Munro, and the aqueduct of Sylvius are dilated. The brain stem appears normal. There is no evidence of change in the posterior lobes.

The brain is softened in the vicinity of the operation.

Cerebellum.—On section, in the left upper part, posterior to the dentate nucleus, is an area seemingly of purulent infiltration of the

tissue, with a matting together of the cerebellar leaflets. The lesion extends into the cerebellar tissue and resembles tuberculosis strongly, but the definite caseation is distinctly absent. The lesion is more diffuse than a tuberculous lesion. On further section this lesion is found to extend posteriorly to the surface of the cerebellum. Another similar but smaller soft mass is found in the right upper posterior portion. It appears like a softening of the tissues, without a definite line of demarcation. Even after hardening it is soft. It extends posteriorly as does the other lesion, and in the posterior part cuts with much resistance, and appears like a meningeal process with extensions into the cerebellum.

No exudate can be seen on the base of the brain.

The spinal cord is removed, but is not observed to be abnormal in gross.

Microscopical Examination.

Cerebellum.—Section through the lower surface with adherent dura. The dura is greatly thickened by dense connective tissue, and is separated from the cerebellar cortex by a wide mass of chronic inflammatory tissue. On lower power observation it is seen that this tissue has areas of irregular shape where there are few cells and much connective tissue; occasionally these areas have an almost spherical form. Between the denser areas the tissue is thickly infiltrated with mononuclear cells. Some areas suggest tubercles; there is a central necrotic or almost caseous mass, with dense connective tissue about it. A prominent element in the tissue as a whole is the large number of giant cells, which are scattered about diffusely, but are more numerous in the denser areas. There are a few small collections of polynuclear cells, slightly larger than giant cells. In giant cells and outside of them can be seen clear spaces, in which small organisms are barely visible.

The cerebellar cortex is partially necrotic and replaced by this tissue, which extends into it in thin strands, so that the molecular layer has a shredded appearance, while the granular layer appears to be intact. High power observation shows that the necrotic areas have in the center caseous cell detritus, in which, especially near its edge, polymorphonuclear cell nuclear detritus and remains of the

organisms to be described later can be made out. Directly border-
ing the necrotic area is connective tissue arranged in general radially,
and containing occasional polynuclear, mononuclear, and plasma
cells; the mononuclear cells here also contain the bodies. Giant
cells are not especially numerous in this zone. Beyond is the main
mass of granulation tissue.

The major part of this tissue is made up of connective tissue which
forms a loose network in the meshes of which occur principally
plasma and giant cells; lymphocytes occur in much smaller numbers,
and polynuclear cells are still more rare, but are seen especially in
small collections about the size of giant cells. Plasma cells with
several nuclei are occasionally found. The giant cells are of two types.
One variety has clear oval or spherical nuclei arranged along the
regular definite border; the other has long spindle-shaped nuclei in
parallel fashion, and long prolongations of the cytoplasm. The most
striking feature of the giant cells is the spaces filled with the organisms.

Organisms.—In size they range from slightly over 1μ to 7.5μ. As
in the preceding case, the large forms are spherical, with a wall about
0.75μ thick. The size of the most frequently occurring organisms
is 4.5 to 5.5μ in the longest diameter; these forms are slightly ovoid,
the diameters of two typical organisms being 4 by 5μ, and 3.5 by 5.5μ;
some forms are so ovoid that they measure 2.66 by 4.66μ. The very
small forms are often much elongated, measuring 1.5 by 2.75μ or
1.87 by 3.5μ. As in the previous case, the wall varies in thickness
with the diameter of the organism, and it is impossible to make it out
in the smallest organisms. They have the same general refraction as
in the previous case; the wall of the large ones is outlined by a double
line, and stains in the same way.

The chief difference between the organisms in this case and the
first case is in the presence of a larger amount of staining material
within the cell; this is to be explained largely on account of the fact
that in the second case tissue was put into Zenker's fluid only 5 hours
post mortem. In sections of the material preserved in formalin there
is the same internal structure as in the previous case; that is, a few
globules or droplets taking many stains slightly. In the Zenker
material stained with aniline blue connective tissue stain, irregular
red-staining masses or specks are seen. In the smallest organisms

these masses are often elongated and slightly curved, and fill more than half the cell space, giving a sickle-like appearance. Often there are two or more separate masses, perhaps connected with a thin thread, or perhaps merely four or five little specks; but no appearance was ever traced out suggestive of a mitotic or amitotic division. In the slightly larger forms the masses occupy a smaller proportion of cell space and show much more variety in shape and arrangement. There may be a circular form with thickenings in the outline; or a long bar with curved cross bars, or a Y-shape or X-shape, or small specks. Occasionally the mass is round or oval, with one or two darker points, and apparently a membrane, and resembles a nucleus closely. Here again no correlation can be made out of changes in the chromatic material with the reproduction processes, except that when budding occurs the large organism sometimes contains the material in small masses, and some of the smallest of these apparently pass out into the bud. More often, a single large chromatic mass, containing practically all the chromatin of the cell, is seen at the budding end. As the bud forms the mass shows a concavity toward the cell interior, and a prolongation of the mass passes out into the bud and then breaks off as the bud separates. This corresponds in general with the changes found by Kohl in torulæ. In the large sized organisms, as in the previous case, chromatic material is slight or absent. The chromatic material stains with hematoxylin and with methylene blue, but as the latter stains the wall the same color it is not so useful for the study of the internal structure. It takes the Marchi stain, and the Scharlach R stain slightly, as in the previous case. With formalin tissue teased out in salt solution, with a drop of 10 per cent sodium hydroxide, the organisms stand out strongly from the tissue elements. Iodine cannot be made out to stain the cell wall.

Reproduction.—Definite buds are much more frequent than in the other case. The medium sized cells are the most active; the process consists of an extrusion of a part of the cell interior at one end, which, when it attains a diameter of 2.5 to 3.5μ, becomes separated from the parent cell by a constriction. The cell wall at first bulges out, then the part enclosing the bud becomes thinner as the bud enlarges, and often appears as a very delicate membrane. Sometimes very small cells are formed, with no visible wall. When small organisms divide,

the bud is more nearly the size of the parent than in the other case. Sometimes a multiplication of the very smallest organisms by a bud the same size as the parent is suggested. The larger organisms bud rarely, but when they do they either send out a protoplasmic mass with an extremely thin membrane or bulge out the whole cell wall. Measurements of budding cells are as follows: a cell 4.25 by 5.5μ is connected by a thin isthmus with a cell 2.25 by 3.75μ; a cell 4.75 by 6μ has just become separated from a cell 2.5 by 3.75μ; a cell 4 by 5μ is connected by an isthmus 0.75μ thick with a mass 1.5 by 2.5μ.

Occurrence of Organisms.—More frequently than in the previous case the organisms partially fill spaces in giant cells, endothelioid cells, or tissue. Often one large organism is seen near the center of a space, with many small ones about the periphery; probably the giant cell took up one organism, which produced another cell by budding, and then by continued budding a small colony was produced, with continued enlargement of the cavity. The original organism becomes large and thick walled, representing an involution form or resting cell. Earlier stages of this process are easily found; in one case a medium sized organism in a giant cell surrounded by a thin space was pushing a bud into the protoplasm of the cell. Occasionally organisms lying in a space are connected by a thread exactly similar to the filaments seen in the previous case, and evidently caused by a drawing out of material about the cells as they separate.

Relations of the Organisms to the Cells.—The organisms have the same destructive effect upon the giant cells as in the previous case. The frequency of budding within giant cells shows even less evidence of ability of the giant cells to harm the organisms. Whether the vacuole in which the organisms lie is a digestive vacuole or whether it represents a lytic effect of the organisms upon the cell cannot be determined definitely. As other tissues are definitely dissolved, and as the vacuole enlarges until the cell disappears, it is possible that it does represent histolysis of the giant cell by the organisms.

There is a definite lytic effect of the organisms upon the cerebellar cortex. Organisms are seen in the outer parts of the molecular layer, and the tissue about them becomes loose and full of spaces, in which plasma cells and then fibroblasts appear.

The cerebellar cortex reacts by the production of a definite gliosis, with heavy glia fibers, which extends into the granular layer. The Purkinje cells next to the lesion show marked chromatolysis and often appear as oval bodies with fine blue granules scattered diffusely through a coarse pink protoplasm, no nucleus being visible.

Sections of the Extensive Areas of Softening within the Cerebellar Leaflets.—There is necrosis of all layers of the cerebellum, with the formation of large masses of necrotic or caseous material and loose collections of lymphoid and large phagocytic mononuclear cells. The latter have finely vacuolated, faintly staining cytoplasm and small round eccentric nuclei, and usually contain organisms. The necrosis often is in great masses, in which few nuclear remains and many hyaline organisms can be made out. There is occasional hemorrhage but no fibrin formation. Large collections of polynuclear cells sometimes border regions of necrosis. Caseous or necrotic areas sometimes touch seminecrotic cerebellar tissue without intervening inflammatory tissue. In a few places, just outside of the cerebellar border, a moderate number of large epithelioid cells are seen. Rarely, next to the cerebellar tissue many necrotic polynuclear cells occur. The cerebellar cortex, in undergoing destruction, becomes vacuolated, especially at the level of the Purkinje cells, the nuclei become further and further apart, and the tissue is more and more indefinite, until it can no longer be recognized as brain tissue. Sometimes there is enormous enlargement of the molecular layer, with separation of nuclei, without definite vacuolization. Mononuclear cells often are present in the vessel walls, and about the vessels there is often a thick plasma cell infiltration. In the midst of the caseous areas vessels often have an intima and media greatly thickened from cell proliferation. There is occasional slight proliferation of the adventitia.

Formation of Large Mononuclear Cells.—The glia cells in the molecular layer become swollen, the cytoplasm faintly staining, and in fine network formation. The cells show some irregularities in form and in distribution, become more numerous near the outer part of the molecular layer, and then crowd the tissue outside. Organisms are often present within them and are apparently destroyed with them, becoming faintly staining and scarcely visible.

Intracerebral Lesions.—Sections from the border of the internal capsule on the right show two lesions between white and gray matter, extending into each, but chiefly into the gray matter. One lesion has two areas of caseation similar to those in the meninges, surrounded by tissue similar to the chronic inflammatory tissue of the meningeal lesion. There is also a spherical accumulation of giant, lymphoid, and connective tissue cells, without central necrosis, containing many organisms in cells and spaces and surrounded by dense connective tissue. The giant cells contain many organisms which destroy their protoplasm, producing spaces which give the dense tissue a vacuolated appearance. There is more dense connective tissue in this lesion than is usual in the meningeal lesion. Where it borders on the brain tissue the lesion is thickly infiltrated with plasma cells and lymphoid cells. The brain tissue near the lesion is either vacuolated or compressed, but shows little cell increase. The other lesion is close to the first and consists of three partly coalescent nodules without necrosis, of similar construction to those just described. In both lesions there is a thick plasma cell layer along the vessels. In neither is there any great infiltrat'on with the large phagocytic mononuclear cells. About both lesions is an increase in glia cells with a slight formation of thick glia fibrils. The ganglion cells show chromatolysis, with eccentrically placed nuclei, and often are indented by glia cells. Occasional organisms can be seen in the loose cerebral tissue about the lesion.

Sections from the left internal capsule also show two lesions separated from each other by nearly normal brain tissue, situated chiefly in the white matter of the internal capsule. The larger lesion has ovoid accumulations, with necrotic polynuclear cells and organisms in the center, and a wall similar to that of the nodules previously described, containing, however, a rather greater number of organisms and showing a more extensive necrosis. Here the organisms are larger and more spherical than elsewhere, are more uniformly distributed throughout giant cells without definite space formation. On the whole, they appear nearly identical with those in the meningeal lesions of the first case. Often each organism has an eccentric red-staining round dot. The giant cells in this nodule are almost entirely converted into thin rims containing a little vacuolated protoplasm

between large numbers of organisms. The tissue about this lesion and the nodule near it is like that previously described. Near the lesion, in the white matter, are ill defined areas, where brain tissue is lacking, and the space is filled with large mononuclear cells. No organisms occur in these areas; they may be areas of degeneration resulting from the lesions or areas in which the organisms have disappeared. There is a slight gliosis about these lesions.

Sections of the cortex from the frontal, temporal, parietal, and occipital regions, and from the uncinate gyrus show, as a rule, no meningitis. There is a peripheral gliosis of moderate degree, very marked in the depths of the sulci. The increase in glia fibers extends almost to the ganglion cell layer, but is marked only in the subpial layer.

In sections of the occipital lobe there is a very marked meningeal thickening in one sulcus. The character of the lesion is in general like that in the cerebellum; but there are more minute collections of polynuclear cells. On one side of the sulcus the inflammatory tissue is making extensions into the brain tissue exactly as it does into the cerebellar cortex, but on the other side there is a very thick peripheral layer of glia fibers, through which no progress is made.

Sections from the temporal lobe show a marked peripheral gliosis, but no meningitis. The ganglion cells here appear slightly shrunken; the Nissl bodies are indistinct, and often a phagocytic glia cell indents the margin. This section also takes in the lateral border of the lateral ventricle, which has a very marked peripheral gliosis consisting of a dense felt of heavy fibers.

The anterior and posterior central regions on the right show edema of the meninges with a slight increase in mononuclear cells, and occasional small organisms. Gliosis and ganglion cell changes are much the same as in the temporal region. The anterior and posterior regions on the left are similar to those on the right. The frontal lobes also have some chronic thickening and edema of the meninges. Gliosis and ganglion cell changes are as in other regions. The uncinate gyrus shows the usual changes. The fourth ventricle and the aqueduct of Sylvius show a heavy gliosis. The choroid plexus appears normal. The spinal cord shows no meningitis. There is no peripheral gliosis.

Weigert stains show interruption of the tracts of the internal capsule on the left by the lesions, with degeneration of myelin sheaths near the lesion, manifested by swollen and bulbous fibers. The internal capsule on the right at the same levels appears normal. The internal capsule lesion on the right is very slight, and interrupts only a few fibers. Sections of the internal capsule at the level of the posterior end of the thalamus show isolated areas of degeneration or absence of myelin sheaths on the left side, the myelin sheaths being replaced by glia cells and fibers. Sections of the spinal cord show a distinct thinning with swelling and degeneration of fibers in the direct pyramidal tract of one side and the crossed pyramidal tract of the opposite side. Sections of the left optic and olfactory nerves appear normal. Bordering the ventricles and in the cerebellum there is degeneration of myelin sheaths, especially near the cerebellar lesions.

Marchi stains show marked deposit of osmic acid in the same areas where the myelin sheath stain showed degeneration. There is also much fat in and about the lesions.

Lungs.—There are large dense fibrous formations of hyaline connective tissue with few nuclei, tending to arrange themselves in large nodular whorls. No tendency is seen toward necrosis or caseation. Bordering the areas are small nodules consisting of lymphoid and endothelioid cell accumulations, with giant cells and connective tissue proliferation, closely resembling tubercles, but lacking caseation. Occasionally there is a slight deposit of brown pigment in the tissue. The near alveoli are filled with plasma and lymphocytic cells, with occasional large phagocytic endothelioid cells and giant cells diffusely distributed. The capillaries are increased in number and size. The giant cells are partially honeycombed with small round or oval spaces, which sometimes coalesce and contain indefinite brown granules in their centers. The appearance is what might be expected as an end-result of an infection of the same character as that of the meninges, but it is possible that it represents merely a form of degeneration. Certain of the giant cells contain radiate or stellate masses lying in a vacuole in the cell which are evidently of the same character as the stellate bodies described by Wolbach in giant cells in the spleen.

Other sections show nodular connective tissue thickenings surrounded by nearly normal alveoli. In these indurated areas are

spherical formations where the tissue is loose and occupied by large irregular giant cells and a few lymphocytes and plasma cells. A later stage shows these nodules entirely converted into connective tissue, with no caseation, but with a surrounding lymphocytic and plasma cell proliferation. In these lesions are a few spherical bodies 4 to 6μ in diameter, with walls 1μ thick, and small central bodies containing central dots. In size and in the character of the wall they correspond with the torulæ found in the brain, but they differ in the absence of the gelatinous capsule and all evidence of multiplication. They probably represent organisms completely obsolete which have become enclosed in the dense connective tissue of lesions which are themselves obsolete. It was the presence of these bodies seen in a frozen section of the lung which first led to the consideration of this case as a possible torula infection.

Bronchial Lymph Node.—Similar connective tissue formations to those in the lung occur in the lymph nodes. Small and large areas of hyaline connective tissue of irregular shape are scattered throughout the node. The first formation of connective tissue takes place about the vessels and in the walls of the lymph sinuses. There are no collections of endothelioid and plasma cells, and no giant cells.

Kidneys.—Slight acute degenerative lesions.

Liver.—One nodular lesion the size of a miliary tubercle consists of giant, lymphoid, and endothelioid cells, embedded in connective tissue with lymphoid infiltration about the periphery.

Spleen.—There are nodular accumulations of endothelioid, lymphoid, and giant cells without caseation. Apparently later stages show hyalinized connective tissue, sometimes slightly infiltrated with lymphoid cells. These lesions usually occur in the follicles, and occasionally can be seen to arise around vessels. In general there is increased connective tissue about the vessels.

Adrenals, Pancreas, Myocardium, and Thyroid.—Negative.

Testicles and Seminal Vesicles.—Normal.

Cultures.—Cultures were made in bouillon of ventricular fluid removed during life. Smears showed round bodies considered to be precipitated stain, or other indefinite material. Nevertheless, the bouillon culture was inoculated into two white mice intraperitoneally under aseptic precautions. About 4 weeks later the mice were

autopsied. Nothing was observed in gross. Microscopical examination of the tissues showed a meningitis consisting of a production of large phagocytic mononuclear cells in which occurred large and small organisms similar to those in the original case. The organisms were multiplying by budding. Some large forms 10μ in diameter occurred. Small forms of 2.5μ were seen. The organisms were nearly spherical, and had a few vacuoles in an otherwise almost homogeneous interior. The large organisms occurred surrounded by wide clear zones, in which radiating lines could be seen in some cases, like those in the first case and in Rusk's cases. In several cases extension into the cortex by solution of tissues without reaction was evident,—early stages of lesions similar to those in the preceding case. A large part of the meninges was edematous, with many organisms, some of them thick walled, but with no cellular infiltration. This is a condition like that of the second case (Rusk's) in the literature. No lesions occurred in other organs.

A. Discussion of Case II.

The chief differences between this and the preceding case depend upon the variations in the extent and activity of the process. In this case the process is so extensive that more necrosis and caseation result; it is so active that budding forms are frequent, and the organisms have not produced the large resting cells or the peculiar brain lesions especially associated in the first case with the presence of the resting cells. The characteristic tendency to solution of tissue is present, the meningeal inflammatory tissue is like that of the preceding case, and the absence of the peculiar intracerebral lesions is made up for by their presence in the brain of the inoculated animal. The difference between this case and those of coccidioidal granuloma or ordinary blastomycosis is obvious; further evidence for its identity with torula will be given under experimental results.

Explanation of the Clinical Course.—Underlying the general pressure symptoms of headache, vomiting, and impaired vision, with the corresponding objective findings, is an internal hydrocephalus, as in the other case. The mechanism here must have been partial blockage of the exit of cerebrospinal fluid from the foramen of Magendie by the dense inflammatory tissue. The hemiplegia is well ex-

plained by the lesions in the internal capsule, partly old and healed, partly active, with the resulting degenerations. The relation of the otitis media to the disease has not been discovered.

The lesions in the lungs, bronchial lymph nodes, liver, and spleen are in all probability due to the action of the same organism. The lesions are unusual, and bodies occur in the lung lesions closely resembling the organisms. Animal experiments show all stages to the production of the cicatricial lesions.

In this case again there is a close resemblance of the lesions with caseation to tuberculosis. There are the same points of differentiation; in this case the predominance of plasma cells is even more marked.

In connection with the data of all the cases of torula infection, it is interesting to observe additional points. Fever was noted in all but the first of the cases in the literature, where its presence or absence is not mentioned, and in Rusk's first case. In our two cases fever occurred only near the time oᶠ onset and was moderate in degree. In all cases where the white count was taken it was not increased, but usually fever was not present at the time. However, in Türck's case the white count was 7,000 when an irregular temperature was run. In ordinary systemic oidiomycosis there is usually a marked leukocytosis, averaging 16,000, and occurring up to 30,000. In our second case and in Rusk's second case there was a positive Noguchi reaction in the spinal fluid. Organisms were found in the spinal fluid in von Hansemann's case; in the spinal fluid and ventricular fluid in Türck's case; and they were evidently present in the ventricular fluid in our second case. In Rusk's cases and in our first case no examination was made. In Türck's case and in our second case cultures did not succeed.

The best means of diagnosis, until some exact serum test is worked out, will be cultures on agar or potato at room temperature, and inoculation of white mice or rats intraperitoneally with spinal or ventricular fluid or cultures, with microscopical examination of the brain. Some ordinary laboratory animals, such as guinea pigs, are practically insusceptible to the disease, and the usual technique of growing cultures for 48 hours on blood serum at incubator temperature must result in many failures; the organism grows much more

surely on agar or potato at room temperature, and growth is often slow, especially after the organism has been passed several times through animals.   Differentiation in smears from tissue elements such as red cells is best effected, as in oidiomycosis, by examining in salt solution or glycerine or with the addition of a drop or two of sodium hydroxide.   In the ordinary stained smears recognition might be difficult.

The symptomatology of the cases resembles closely that of brain tumor.   The essential factor in the similarity is the occurrence of a chronic, slowly progressing inflammatory process, which by being situated in such a way as to interfere with the passage of cerebrospinal fluid causes an internal hydrocephalus, or results in the formation of masses of parasites in various parts of the cerebrum or cerebellum, or causes a chronic meningitis with overproduction of fluid. Involvement of special nerves may give false localizing symptoms. The absence of cutaneous or recognizable systemic lesions adds to the confusion.   The pathological reports of other cases of pseudotumors are so scanty that it is impossible to tell whether many of them may not have the same or a similar explanation.   Pseudotumors have been one of the mysteries of brain surgery; an explanation of a few cases now gives a definite line of attack for future work.   Necessarily there are also points of similarity with chronic diseases of the nervous system, such as syphilis.

IV.   CLASSIFICATION OF THE DISEASES FORMERLY CALLED
BLASTOMYCOSIS.

Botanically torulæ form a group of organisms similar to the yeasts in the form and organization of the cell and the method of reproduction by budding, but different in the constant absence of endospore production and the frequent lack of power to ferment sugars.   Other points of difference are commonly present, but not invariably.   From the oidia they are distinguished by their absence of mycelium production under all circumstances.   The true yeasts are classed in most systems as ascomycetes on account of the endospore production. The real biological relationships of the yeasts and torulæ are not yet determined.   The type of organism budding in tissues and pro-

ducing mycelium in cultures is botanically an oidium. The oidia have no characteristic fructification and hence their systematic position is also obscure.

Coccidioides immitis is definitely an ascomycete on account of its endosporulation, and is seen to be in close biological relation to the yeasts.

The various organisms belong to different groups and have different appearances and pathological effects. Coccidioidal granuloma is already established as a name for the disease produced by the ascomycete with hyphæ, and causes no confusion. The term blastomycosis, however, which signifies a disease caused by a budding organism, obscures the real differences in the various diseases which have been included under its name, and should be discarded. Oidiomycosis, yeast infection, or torula infection should be substituted, according to the case. Following is a summary of the differential points in torula infection, oidiomycosis, and yeast infection.

Summary.—1. True yeast infection, produced by an organism which reproduces by budding. It produces no true mycelium, forms endospores under certain conditions, and usually ferments sugar. The organism is rather feebly pathogenic for animals. There are two authentic cases in man, the first being Busse's. Both cases include skin lesions. The pathological changes are characterized by necrosis, stimulation of epithelial growth, some exudation of polynuclear cells, tendency to giant cell formation, nodular accumulations of small round cells, and abscess formation. The organism has an adventitious capsule occasionally in human and animal lesions, but does not produce a gelatinous matrix and does not have even a capsule in brain lesions in animals.

2. Torula infection, produced by a yeast-like organism, distinguished from yeasts by its absence of endospore production under all circumstances. It never produces mycelium, usually does not ferment sugar, and reproduces by budding alone. The organism is more pathogenic for animals than that of the preceding group. The first spontaneous case reported was described by Frothingham as occurring in a horse. Six cases are known in human beings. In localization the nervous system especially is affected, to a less extent other organs, never the skin as part of the general infection. The symptoms

are chiefly caused by the brain lesions. The pathological changes in the meninges are characterized by chronic inflammatory reaction, with areas of caseation like tuberculosis, if the lesion is extensive; the organisms usually occur in cells, bud freely in the large vacuole enclosing them, and usually destroy the cells. The organism produces peculiar intracerebral lesions, consisting of a lysis of the cerebral tissue, with only a very chronic proliferative reaction which is often almost lacking, and fills the lesion with a peculiar gelatinous material holding the organisms separate, in which occasionally threads and star-like processes form. In other organs it produces, as a rule, nodular lesions resembling miliary tubercles which finally become dense connective tissue masses. The organism in tissues has a nearly homogeneous, non-granular, faintly staining interior except in the smaller forms, varies in size from 1 to 13μ, and often produces large resting cells. The wall stains easily and diffusely. The organism increases by budding. Small buds may be produced by organisms of any size. When produced by an organism of small size they may equal the size of the parent. The small forms have a good deal of chromatic material in masses. In mice and rats pure cultures produce lesions of the same character as those found in man.

3. Oidiomycosis is caused by an organism reproducing by budding in tissue, and forming mycelium in cultures. There is no endospore production. There are over thirty recorded cases of systemic involvement, and more numerous cutaneous cases. In localization oidiomycosis affects especially the skin and subcutaneous tissues, no reported systemic cases being free from such involvement; it often involves bones; it affects all internal organs, including the brain, but has not yet produced lesions in the brain which have caused symptoms until just before death from the general infection. The pathological changes are characterized by necrosis and overgrowth of epithelium with miliary epidermoid abscesses, forming characteristic skin lesions; abscess production is present in deep tissues and in organs, also tubercle-like nodules; there is a marked attraction for leukocytes (except in occasional cases in the spleen and bone marrow); no lesions have the peculiar gelatinous matrix (although occasionally, especially in the bone marrow, an adventitious capsule is produced). The only reported case of meningitis was a purulent one; the brain lesions are

much like the other lesions with somewhat less reaction.   The organism in tissues has a granular interior, usually with a separation of
the protoplasm from the wall by a distinct space, is spherical, and
reproduces by budding.   The wall stains with difficulty or not at
all.   It is from 3 to 30μ in diameter, the average size being 16μ.
In animals it produces lesions with difficulty; they resemble the
lesions in man.

V. EXPERIMENTAL.

1. Materials and Methods.

All inoculations were made with pure cultures suspended in sterile
salt solution.   The organisms with mycelium were teased into as fine
particles as possible.   The site of each inoculation was shaved, washed
with alcohol, and painted with iodine.   We used the following
organisms: a·culture of torula from Frothingham's case of infection
in a horse, a culture of oidiomycosis from the human cutaneous case
reported by Wolbach, and a culture of *Coccidioides immitis* from a
human case of coccidioidal granuloma.

Since so much experimental work has been done on so called
blastomycetes and *Coccidioides immitis*, our results, which confirm
the differences in the two organisms, will be given with especial reference to the histological changes rather than to the morphology of the
organisms in the tissues.

2. Experiments on Dogs.

A thick suspension of each organism was injected into a dog intravenously to the amount of 5 to 8 cc.   Later a similar suspension was
injected into the lower bronchi through a rubber catheter introduced
into the trachea.   The animals were killed in 3 to 6 weeks.   The dogs
inoculated with oidiomycosis and coccidioidal organisms showed no
lesions.   The animal inoculated with torula had no lesions in any
organs but the brain and kidney.   The kidney showed a few minute
white dense spots, about 1 mm. in diameter, in the medulla.   On
serial section of the brain hardened in formalin several small white
areas 1 to 2 mm. in diameter were found in the cortex, white matter,
and basal ganglia.

Microscopical Examination. Kidney.—The lesion consists of a diffuse collection of lymphoid and plasma cells with a few polynuclear cells, obliterating and replacing tubules. Plasma cells predominate. Other lesions consist of a diffuse connective tissue formation, with areas of plasma cells. No definite organisms can be found in these lesions. The lesions resemble closely those in the kidney in the first case.

Brain.—The lesions consist of focal collections of mononuclear cells with rare polynuclear cells. Some of the cells are plasma cells; most of them have a large amount of cytoplasm and large round centrally located nuclei, and appear to be arising from the glia cells about the lesion. A few large mononuclear cells are seen. Polynuclear cells are extremely rare. Some of the lesions are near vessels, and about the vessels there are dense masses of lymphoid cells. A few partially disintegrated organisms are found in these lesions.

These few experiments show that the dog must be regarded as a relatively resistant animal to the three groups of organisms. It is interesting that the only lesions in the series of dogs are brain and kidney lesions from the torula injection, and of these the brain lesions are the most numerous.

3. Experiments on Rabbits.

One series of rabbits was inoculated intraperitoneally with 2 cc. of a thick suspension of each organism, another intravenously in the ear vein, one subcutaneously, and one subdurally. With oidiomycosis no lesions were produced. The subdural inoculation with the coccidioidal organism resulted in an abscess at the site of inoculation from which a pure culture of the *Coccidioides* was obtained. In the rabbit inoculated intraperitoneally with the torula, lesions were found in the kidney, brain, and meninges.

Rabbit 1.—Intraperitoneal inoculation with torula. Animal autopsied 3 weeks later. In gross no lesions were noted.

Microscopical Examination. Liver.—A large area is found nearly the diameter of a low power field, with central caseation, surrounded by epithelioid cells, then a zone of thick infiltration with lymphoid

cells, containing many eosinophilic cells, with a slight surrounding connective tissue band. No organisms are found in the lesion.

Brain.—A chronic meningitis in which definite torula organisms are found. The cells consist chiefly of large mononuclear cells and sometimes contain organisms. There are moderate numbers of plasma cells, but no polynuclear cells. The organisms are scarce and show much degeneration. The large phagocytic mononuclear cells are filled with hyaline droplets or vacuoles. The cortex near the lesion has a slight increase in glia tissue, with spider glia cells. In the cortex are focal lesions consisting of accumulations of mononuclear, lymphoid, and plasma cells, the lesions being about the diameter of a high dry power field. Organisms occur in these lesions but are difficult to find. The meningitis is not found in all sections.

Rabbit 2.—Subcutaneous inoculation with *Coccidioides immitis.* Autopsy 7 weeks later. A nodule 1 cm. in diameter was found at the site of inoculation open, with a thick purulent content. No internal lesions. Sections of the nodule show diffuse connective tissue increase and many large irregular masses of polynuclear cells, with little necrosis.

The intervening connective tissue is remarkably free from cellular infiltration. The abscess contents consist practically entirely of polynuclear cells. The border is sharp and made up of a band of denser connective tissue. Caseation occurs near the borders of the abscesses, rather than in the contents. No giant cells are present. The earliest lesions consist of small accumulations of polynuclear cells with surrounding connective tissue proliferation. The organisms occur in the abscess and in the connective tissue. They are placed close to the cells without intervening spaces.

Rabbit 3.—Subdural inoculation with *Coccidioides immitis.* At the site the meninges are greatly thickened with increased connective tissue and abscess formation. The lesion has extended into the cortex and is bordered there by a zone of connective tissue thickly infiltrated with plasma cells and lymphocytes. Outside of this connective tissue band polynuclear infiltration becomes more marked and large abscesses are found similar to those in the subcutaneous nodule just described, but more extensive, and with more necrosis and peripheral caseation. Giant cells occur frequently at the borders of the abscesses. The organisms occur in giant cells and free, but they never have a clear zone about them. They are seen more often outside than in the abscesses. Sporulating forms are rare. One large abscess has several free pieces of cortex within it. In the series of rabbits the tendency of the torula to infect the nervous system is evident. In one case a meningitis is produced from intraperitoneal injection. The coccidioidal organisms produce brain lesions only from subdural inoculations.

4. Experiments on Guinea Pigs.

Inoculations were made intraperitoneally and subdurally. No lesions were produced with oidiomycosis. With torula the only lesions produced were peritoneal nodules in the guinea pig inoculated intraperitoneally. The surface of the peritoneum was slightly dull and dotted with minute, raised, slightly whitish areas, appearing much like a culture of streptococcus on agar. Microscopically the lesions consisted of lymphoid and endothelioid cells in connective tissue with a few mono- and polynuclear eosinophils. The large resting cell forms of the organism occurred in the nodules singly, each organism surrounded by a clear space, about which were endothelioid cells which showed signs of fusion into giant cells. The muscle fiber just underneath the nodules showed a loss of the cross-striation so plain elsewhere.

Two of the guinea pigs inoculated with the coccidioidal organisms had lesions; the one inoculated subdurally had a brain lesion.

Guinea Pig 1.—Inoculated intraperitoneally with *Coccidioides immitis*. At autopsy abscesses are found in the testes, epididymis, and liver, and nodules in the peritoneum. There are no lesions in the central nervous system. The lesions consist of abscesses, large and small, and collections of epithelioid cells, as in the preceding case. The small circumscribed collections of polynuclear cells about a few organisms are frequent. Caseation is always in the zone about large collections of polynuclear cells. In the giant cells the organism enlarges until it finally appears closely surrounded by a thin band of the protoplasm and nuclei which appear unchanged. Organisms sporulate much more often than in the rabbit, and the leukotactic influence of the young organisms is very plain.

Guinea Pig 2.—Inoculated intraperitoneally with *Coccidioides immitis;* killed in 4 weeks. Lesions in the peritoneum, testes, epididymis, liver, spleen, and lung. There are no brain lesions. Pure cultures of the coccidioidal organism recovered from the testicular lesion. The lesions are similar histologically to those in the previous animal.

Guinea Pig 3.—Inoculated subdurally with the coccidioidal organisms. At autopsy an abscess is found at the site of the inoculation, from which a pure culture of the coccidioides was recovered. No lesions in the other organs.

Microscopical Examination.—Large and small masses of polynuclear cells, with surrounding connective tissue often infiltrated with polynuclear cells, have greatly thickened the meninges in and about a sulcus, compressed, and in one place invaded the surrounding cortex. A

single mass of polynuclear cells is one and one-third times the diameter of a low power field. Organisms are numerous except in the thick connective tissue. Giant cells are rarely seen. Some small masses of polynuclear cells are surrounded by a zone of epithelioid cells. Where the lesion has eroded the brain there is much new vessel formation with exudation of polynuclear, plasma, and lymphoid cells, and in the brain all about the lesion there are polynuclear, plasma, and glia cells, sometimes forming thick masses. The meningitis is localized to the neighborhood of the sulcus.

5. Experiments on Mice.

Lesions were produced with the coccidioidal and the torula organisms, but not with that of oidiomycosis. The culture of oidiomycosis had been used previously in animal experiments, but had probably lost its virulence with time. The torula culture, which was just as old, seemed to have retained its virulence extremely well, for general lesions were easily produced in mice and rats. We were able, through the kindness of Dr. S. B. Wolbach, to procure paraffin blocks of the animals used in his experiments with oidia. The lesions found will be described briefly.

Coccidioides immitis Lesions.

Mouse 1.—Inoculated intraperitoneally with *Coccidioides immitis*. At autopsy a pure culture of the organism was obtained from a nodule in the liver and from a peritoneal nodule at the site of injection. Lesions were found in the mesentery, peritoneum covering the liver and spleen, and in the lung and liver.

Microscopical Examination. Lung.—Nodules consisting of many small organisms mingled with polynuclear cells. One mass consists of large organisms nearly all of which are sporulating, surrounded by a hemorrhagic zone containing a few polynuclear cells. Some lesions show little reaction other than hemorrhage; others large numbers of polynuclear cells and few organisms. Usually there is a beginning invasion of polynuclear cells and formation of connective tissue.

Liver.—There is a small collection of organisms with an early invasion of polynuclear cells. The liver tissue about the colony is compressed. Other larger lesions show diffuse infiltration with polynuclear cells and a connective tissue capsule. The liver cells

immediately surrounding the colony appear normal except for compression. Other lesions consist of small abscesses containing small organisms and without surrounding connective tissue. Adherent to the peritoneal surfaces of the liver, spleen, and pancreas are large and small organisms with varying amounts of acute exudate. The mesentery is thickened with a purulent exudate and many organisms. There is a good deal of new vessel formation, and an infiltration in these places with plasma cells. There is necrosis, almost caseation, of organisms and cells in a diffuse manner. Peritoneal nodules occur with polynuclear cells, especially about small organisms.

The chief peculiarity of the lesions is the greater size of the large organisms, some of which measure 85μ in diameter, and the great frequency of sporulation, some lesions consisting of numbers of large sporulating cells producing masses of small organisms. The small amount of reaction about some lesions in the liver and lung seems to be due to the recent invasion of the tissue, for an acute reaction with hemorrhage is always present to some extent. Most of the lesions are fairly characteristic except for the small number of epithelioid and giant cells. The coccidioidal organism is a virulent one, and the infection evidently overwhelmed the animal.

Lesions with an Oidiomycosis Organism.

Lungs.—The bronchi contain a purulent exudate with organisms and the walls are necrotic. Lobules of the lung are solidified with enormous numbers of oidia and a purulent exudate. It is difficult in these areas to make out the alveolar walls, which frequently appear to have been destroyed. Occasional small irregular areas of caseation are seen immediately surrounded by the infiltrated tissue and apparently the result of the crowding of the enormous numbers of the organisms in the tissue. The organisms lie close to each other or to the polynuclear cells, without intervening spaces. They are occasionally seen budding. Large endothelial cells occur and contain organisms, which distend the cells, but do not seem to injure them otherwise than by this mechanical action. There are a few giant cells in which organisms occur without causing solution of their protoplasm. No connective tissue formation is evident. Other large

areas, ·normal except for a few large endothelial cells, immediately adjoin these lesions. About some of the vessels there is a plasma cell infiltration.

Lesions with Torula Organisms.

Mouse 2.—Inoculated intraperitoneally with torula. The peritoneum contains a slight amount of sticky exudate; the surface is dull, and small white thickenings are visible on the surface. The omentum and mesentery are thickened. No lesions can be found in the internal organs, except the brain. Pure cultures were obtained from the peritoneal exudate.

Microscopical Examination.—In the peritoneum are enormous numbers of very minute organisms and a few larger ones, appearing as empty shells which are often broken open. There is an infiltration with a few mononuclear cells and a moderate number of eosinophils. The organisms occur in spaces, some of which are fat spaces, others formed from destroyed cells. These spaces give the tissue a honey-combed appearance. In the omentum is a similar lesion with a slightly greater reaction, consisting of numbers of lymphoid, plasma, and large endothelioid cells. The brain shows a meningitis consisting of a considerable accumulation of large mononuclear cells in which no definite torula organisms can be found.

6.   Experiments on Rats.

Inoculations were made intraperitoneally with the torula and oidiomycosis organisms. No lesions were produced with the oidiomycosis, but with the torula a general infection with lesions in the meninges, brain, and cerebellum was produced. Pure cultures of the organism were recovered from the animals in nearly all cases.

Rat 1.—Inoculated intraperitoneally with 2 cc. of a thick suspension of torula on Feb. 24, 1915. The animal died on Apr. 14, 1915.

Autopsy.—The peritoneal cavity contains no fluid, there are no adhesions, and no nodules on the peritoneum. The surface seems slightly dull and sticky. The abdominal organs and lymph nodes appear normal. The lungs are greatly distended, firm, pale grayish pink, with a slightly nodular appearance of the surface. All lobes of both lungs are affected in equal degree. On section the surface is whitish and sticky and slightly nodular. The tissue has a semitranslucent appearance. The brain appears normal.

Smears from the peritoneal cavity and lung show only torula organisms. In the smear from the lung there is much homogeneous material adherent to the

organisms, staining faintly and obscuring the details. A culture from the peritoneal cavity gives a pure growth of torula. A lung culture gives a growth of torula, contaminated with other organisms.

Microscopical Examination. Lung.—The alveoli are dilated and often broken to form large spaces, which are filled with a gelatinous material in which organisms are embedded. With material fixed in Zenker's fluid the gelatinous material stains a faint reddish blue and is seen to be filled with fine granules taking a light red stain. There are irregular thickenings which stain blue and often take the form of threads joining the organisms. As a whole, the substance has shrunken during fixation, so that irregular spaces exist in it. With formalin fixation and hematoxylin staining the material is scarcely colored at all, but occasionally stains slightly where it is thickest. Cresyl blue after formalin fixation colors it intensely, however, and shows fine radiating thickenings from the large organisms. The larger organisms are spherical, 10 to 13μ in diameter, with a definite wall staining diffusely with methylene blue, which is often irregular in outline as if somewhat collapsed. The contents are homogeneous and stain faintly. The whole general appearance of the lesion and organisms is identical with that of the peculiar cerebellar and cerebral lesions of the first case. On close examination enormous numbers of small organisms varying in size from 1 to 6μ in diameter are seen. These occur especially in spaces surrounded by fragments of the destroyed cells of the alveolar walls. The method of reproduction is always by budding; medium sized organisms give off small buds 1 to 2μ in diameter, and organisms 3 to 4μ give off the smallest forms. Occasionally, the small forms appear to reproduce by buds the size of the original organism. These small forms are more oval than the larger ones and appear exactly like those in the second case except that they are not quite so thick walled and are not so frequently seen budding. They show the same relations to cells, multiplying freely in large vacuoles in giant cells, and finally destroying the cells. They have clear zones about them. A cresyl blue stain colors the outer surface of these organisms and shows that even the smallest has a thin membrane or wall. With hematoxylin the wall of the small forms scarcely stains, so that one sees principally the chromatic material, and an impression is gained of extreme minuteness. In the spaces the

organisms frequently occur as one large organism, and many small ones, the latter frequently about the periphery of the space. In spite of the enormous numbers of small organisms in contrast to the fewer larger ones, no evidence of a process of endosporulation is found. The only suggestion of it is the finding once of a large cell with many small chromatin masses of varying size irregularly distributed in the cell. Frequently the larger organisms are seen giving off the minute forms as buds.

The reaction in the lung is very slight, and consists of a perivascular exudate of plasma and lymphoid cells, with production of large mononuclear and giant cells containing organisms. Occasional small hemorrhages are seen. In some areas there is a formation of dense connective tissue about the organisms, forming nodular masses, infiltrated slightly with lymphoid and plasma cells. Other sections show areas of diffuse production of connective tissue.

Spleen.—A lesion extends into the pulp from the surface. It consists of a formation of dense connective tissue with a loose meshed tissue on the peritoneal side enclosing spaces filled with organisms. The organisms are nearly all of the large resting cell type. They show signs of degeneration, and reproduction is not active. In another place there is a formation of dense connective tissue on the peritoneal surface of the spleen with a number of mononuclear eosinophilic cells and a few barely recognizable organisms.

Kidneys.—A number of small irregular areas of dense connective tissue contain a few thick walled organisms. Sometimes an area encloses an atrophic glomerulus. One area of dense connective tissue extends in irregularly from the surface. Honeycombed spaces in the center contain hyaline organisms. In one place dilated tubules with atrophic epithelium contain organisms, and nearby tubules on close examination show small organisms in the epithelium, causing its destruction. About the infected areas are a few lymphoid and plasma cells.

Liver.—A lesion extends in from the surface, consisting of a caseous center containing large organisms, then a layer of giant cells and epithelioid cells containing organisms, beyond which is a thick wall of connective tissue.

Brain.—A chronic meningitis is found in all sections, varying some-

what from a slight cellular infiltration to a marked thickening. Large numbers of small and fewer large organisms are present everywhere. The cell reaction consists of lymphoid and plasma cells, and a few giant cells, but principally of large phagocytic mononuclear cells. Rare polynuclear cells occur. Connective tissue formation is beginning.

The organisms always occur with clear zones about them, demonstrated by special stains to consist of a gelatinous material. There are extensions into the cortex forming lesions with solution of tissue filled with gelatinous material, and absence of reaction, exactly like the peculiar brain lesions in the first case. The only difference is in the great number of small organisms present in the large phagocytic mononuclear cells which have invaded the lesion. The small organisms are seen in the substance of the cortex and the edge of the lesion, causing its disintegration. In some of the lesions there is considerable production of large phagocytic mononuclear cells which are filled with the small organisms; in other lesions these cells are few. Cresyl blue stains the gelatinous material in the lesions as in the first case. There are several lesions in the cerebral cortex, and in the cerebellum.

The small organisms occurring in large numbers are in contrast to the lesions of the case described by Frothingham, from which the organism was obtained. Evidently, as in the coccidioidal disease, where enormous numbers of sporulating forms were found in the lesions in mice, the small animals furnish a particularly favorable ground for their multiplication.

Rat 2.—Inoculated Apr. 25, 1915, by an injection of 1 cc. of a salt solution suspension of torula into the right pleural cavity; killed May 15. No external lesions. The peritoneal cavity appears normal. A smear shows no organisms, and cultures give no growth. Small white spots, 1 to 2 mm. in diameter, are found in the liver and kidneys. The spleen and lymph nodes appear normal. Both lungs are filled with gray nodules about 1 mm. in diameter. The brain appears normal externally.

Microscopical Examination. Lung.—Scattered throughout sections of the lung are miliary nodules which often occur in groups of two to ten, like miliary tubercles, the adjacent nodules often coalescing to form larger ones; these are composed of collections of

epithelioid cells with a few lymphocytes and occasional giant cells. About the periphery of the nodule there is slight connective tissue formation, and there are often numbers of eosinophilic cells. The alveoli next to the nodules appear normal. In some of the nodules torula organisms can be found. They occur in the center, often with a few eosinophilic cells, and show all stages of degeneration to complete destruction. The majority of the nodules contain no intact organisms; in some not even remains of organisms can be made out. These nodules are indistinguishable from miliary tubercles, produced by the tubercle bacillus, except for the presence of the organisms. In some cases there is a slight central caseation, but usually none is present. Giant cells vary much in numbers; sometimes six or seven occur in a section of a nodule, in others there are none. Although they often contain the organisms, their number does not seem to be in relation to the numbers of the torulæ.

Spleen.—Tubercles similar to those in the lung, but of larger size and with central caseation, are found. In some nodules remains of the small forms of the torulæ but no large forms can be seen.

Peritoneal Nodule.—There is a diffuse chronic inflammatory tissue very much like that of the meninges with lymphoid, plasma, and epithelioid cells. Eosinophilic cells are scarce as a rule, but are often numerous about vessels. Giant cells occur diffusely; a few organisms, in a partially disintegrated condition, are seen.

There are large and small coalescent areas of necrosis with partial caseation in which torulæ are numerous. The caseous material is partially honeycombed with spaces, in the centers of each of which one of the larger forms of the torulæ is seen. Remains of cells can often be seen in the caseation, and fibrin is often present at its periphery.

The whole picture is similar to an advance tuberculous node, except that the caseation is not complete and there are often visible torula organisms.

Kidney.—Miliary collections of epithelioid and giant cells as in the lung.

Liver.—Perivascular collections of lymphoid, plasma, and many eosinophilic cells, and a few miliary nodules similar to those described above.

Brain.—A chronic meningitis in some of the large sulci with extension into the cortex. The whole picture is just like that in the rat previously described, except that the organisms are undergoing greater destruction and the lesion is being invaded by the chronic inflammatory tissue from the meninges. This shows the chronic recoverable character of the brain lesions caused by the torulæ.

Rat 3.—Inoculated Apr. 25, 1915, by an injection of 1 cc. of a salt solution suspension into the heart. The culture was obtained from the peritoneal cavity of Rat 1. On May 15 the animal was noted to be sluggish in reaction and to keep in a crouching position with somewhat labored breathing. Gradually the symptoms became more marked and finally the animal no longer attempted to defend itself when touched, and when rolled over righted itself with difficulty, and walked slowly with a dragging gait. The hind legs seemed weak, and the animal could not run.

On May 25, 4 weeks after inoculation, the animal was chloroformed and autopsied. The peritoneal cavity appears normal. Many small gray nodules are found in the kidney, from 0.33 to 1 mm. in diameter. A few very small gray nodules in the spleen appear to be different from the Malpighian bodies. No lesions in the liver. The retroperitoneal nodes appear normal. The lungs are a little more firm than normally, and contain many nodules similar to those in the kidney. In the mediastinum is a thickened fibrous mass of tissue in which enlarged lymph nodes are found containing miliary lesions. The brain is considered to have a few minute nodules, but their presence is not certain.

Cultures on potato and dextrose agar from the meninges, and from the lung and kidney lesions give pure growth of torula.

Microscopical Examination. Brain.—In five sections from five different blocks of the cerebrum and cerebellum forty-two lesions are found in the brain substance, and many areas of infection of the meninges. The lesions in the brain are perivascular or extensions from the meninges. They are all in a stage of healing, and show a high degree of destruction of the parasites. Sometimes in a lesion are only one or two large collapsed organisms, or there may be a small collection of the large non-granular spheres. The small forms so conspicuous in the rat that died from the injection are almost entirely absent. The method of extension of the brain lesions by a solution of the tissue by the individual organisms is plain. The reaction in all is of an extremely chronic character. There are accumulations of plasma and lymphoid cells with a few large mononuclear cells in the perivascular lesions. Almost all the reactive cells in the lesions with

solution of tissue are the large mononuclear cells, probably glial in origin. The meningeal reaction is similar to the perivascular. Many lesions are present in the cerebellum, especially in the cortex. There is a distinct production of thick glia fibers in the outer layer of the cerebral cortex. The whole picture here is strikingly similar to our first case. A lesion is found in the choroid plexus of the third ventricle consisting of an ill defined accumulation of mononuclear cells. The choroid plexus of the fourth ventricle contains an almost perfect imitation of a miliary tubercle, without caseation.

Kidneys.—Numerous single and conglomerate tubercles are present, some with a few hyaline or collapsed large torula forms, others in which none can be made out. In several instances large thick walled organisms are seen in glomeruli. The character of the tubercles is the same as in Rat 2. There is occasional necrosis in the center, with remains of organisms, the material staining a nearly uniform pink and resembling caseation. Occasionally there is a diffuse infiltration with lymphoid cells between the tubules.

Liver.—Many small lesions are seen, consisting of collections of lymphoid cells about small masses of hyaline organisms in the periportal tissue. Sometimes dense connective tissue in considerable degree is formed.

Spleen.—Frequently in a large giant cell eccentrically placed in a Malpighian nodule are numbers of small torulæ.

Lungs.—Numerous miliary nodules as in Rat 2. More often recognizable organisms can be seen. .

Lymph Nodes.—(Mediastinal.) Numerous and large lesions like those in the peritoneum in the preceding case. The reaction is almost entirely of epithelioid and giant cells with formation of connective tissue. Often a ring of connective tissue encircles the clear zone about an organism. There are also miliary lesions like those previously described. Some of the lymph sinuses are distended with organisms which have not yet excited a reaction. .

Stains for tubercle bacilli were made, but no organisms found in any of the tubercles.

Rats 4 and 5.—Inoculated intraperitoneally on Apr. 25, 1915, with a few drops or a thin salt solution suspension of torula from the original culture. 2 weeks later inoculated intraperitoneally with 2 cc. of a thick suspension of torula obtained from Rat 1. No symptoms of illness. Killed May 25.

No lesions in gross except in the retroperitoneal lymph nodes, which are enlarged, with miliary white areas, and in the peritoneum covering the spleen and kidneys.

Cultures from the peritoneal cavity, heart's blood, and brain give no growth.

Microscopical Examination.—The absence of lesions in the organs is confirmed. The lesions in the retroperitoneal nodes are extensive and of the type already described.

Rats 6 to 18.—Inoculated intracardially on June 22 with 2 cc. of a salt solution suspension of torula recovered from Rat 1. Rat 6 died on the 2nd day, Rats 7 to 10 on the 5th day, Rat 11 on the 6th day, Rats 12 to 14 on the 7th day, Rat 15 on the 9th day, Rat 16 on the 10th day, Rat 17 on the 11th, and Rat 18 on the 12th day. Symptoms of illness consisting in lack of vigor and alertness preceded death by 1 to 2 days. At autopsy pneumonia and pleurisy of the type already described were found in all the rats except Nos. 6 and 9. In gross the only other lesions noted were enlargement of the spleen and lymph nodes.

Pure cultures of torula were recovered from the spleen and liver of Rat 6, from the spleen of Rat 12, and from the spleen and meninges of all the other rats.

Microscopical Examination.—Brain lesions are seen in every case, besides miliary lesions in other organs, which are sometimes extensive. The type of reaction is that previously described. In no case can an acute exudate be found. In the brain lesions reaction is practically completely lacking, even though a considerable amount of reactive tissue exists in the other organs. The brain lesions are smaller than in the rats killed later, while the lesions in the other organs are, as a rule, more extensive. Apparently the torula grows faster, but excites more resistance in most organs, while in the brain it grows slowly but keeps on for a longer time.

Control Examination.—Six uninoculated rats from the same lot were autopsied and examined histologically to be sure that similar diseases did not occur spontaneously in the animals. No lesions were found.

7. *Agglutination Experiments.*

Agglutination tests were done on sera taken from all the inoculated animals. In the first series blood was taken from the heart on Jan. 17, and the sera were kept in the refrigerator. On Jan. 18 a suspension of the torula organism was tested against the sera in dilutions of 1:20, 1:100, and 1:1,500. The tubes were put in the incubator and

readings made in $\frac{1}{2}$ hour and $2\frac{1}{2}$ hours; the tubes were then kept at room temperature, and a third reading was made at the end of 12 hours. All tubes showed the same degree of clearing, with sediment of similar appearance. On Jan. 19 a torula suspension was tested against the sera again in dilutions of 1:2, 1:10, 1:1,000, and 1:5,000, and readings were made as before. All tests were negative.

On Jan. 22 oidiomycosis and coccidioidal organisms were similarly tested with all the sera, and no agglutination was found in dilutions of 1:2, 1:20, 1:100, and 1:500.

All tests were then repeated in dilutions of 1:2, 1:20, 1:100, and 1:500, and found negative. In all tests salt solution and sera of normal rabbits and pigs were used as controls.

Of the animals tested, four were found at later autopsy to have lesions produced by the torula; one rabbit, one dog, one guinea pig, and one mouse. Of the animals inoculated with oidia, none had lesions. Five animals had coccidioidal lesions,—three guinea pigs, two rabbits, and a mouse.

In the second series macroscopic and microscopic agglutination tests were done on the sera of the mice and rats infected with torula and oidiomycosis. The rats infected with torula proved later to have extensive lesions. First a suspension of the torula was tested against the sera of two mice infected with torula and one rat infected with oidiomycosis in dilutions of 1:20, 1:30, 1:90, 1:180, 1:540, and 1:620. The readings were made as before. No definite agglutination could be made out.

The microscopic tests were made in dilutions of 1:10, 1:30, 1:90, 1:180, 1:360, and 1:720, and were examined at intervals for 18 hours. No agglutination occurred. The microscopic tests were repeated with negative results.

Later it occurred to us that possibly the gelatinous secretion of the torula interfered with the physical conditions necessary for the test, as in the case of Friedländer's bacillus and other encapsulated bacilli. Consequently treatment with acid, according to the method of Porges and Prantschoff, was tried. Dilutions were made of 1:2, 1:50, 1:100, 1:200, and 1:500. After 2 hours in the incubator complete agglutination was found in the sera of one mouse in dilution of 1:50, partial in dilution of 1:100, a questionable slight clumping in dilution

of 1:150, and none in 1:200. This test occurred in a mouse which was autopsied at the time blood was taken and found to have a sticky peritoneal exudate from which a pure culture of torula was obtained. The test was made about 3 weeks after inoculation. The other mouse gave slighter agglutination. This mouse had been inoculated only 3 days previously, but at autopsy at the time of the experiments had a peritoneal exudate containing a pure culture of torula. Careful control tests with normal rat blood, however, showed practically the same degree of agglutination. The tests were also carried out by this last technique on the blood of Rats 3, 4, and 5, taken at the time of autopsy, and the same results obtained.

Conclusion.—No agglutination test of diagnostic value occurs in an infected animal.

8. Cultural Characteristics of Torula.

We repeated and confirmed the observations previously reported in regard to Frothingham's torula. Observations were made on cultures growing under varying conditions of temperature and moisture on many media. Direct observations of growth were made on agar slides and in hanging drops. Reproduction was always by budding. In cultures fresh from animals the production of small forms about 1.25μ in diameter occurred.

Fermentation tests in 1 per cent lactose, saccharose, dextrose, dextrin, and glucose, in Smith fermentation tubes, resulted in no gas production.

No spore production was found in cultures or in our observations of growth on gypsum blocks. Extensive observations of gypsum cultures had already been made by Weis.

The growth at first was most abundant on carbohydrate media,— potato and agar or dextrose agar,—and was extremely scanty on blood serum. After animal inoculation growth occurred more vigorously on blood serum, but never so abundantly as on the dextrose agar or potato tubes. No growth or very slight growth occurred in anaerobic cultures. Growth first occurred in 12 hours to 4 days. It was nearly as vigorous at room temperature as in the incubator. The colonies are first white, then they acquire more and more of a yellow tinge as they grow older. Colonies starting from a single organism

become much heaped up, probably from the adhesive nature of the capsule. In the usual tube inoculation a smooth, pasty, slightly shiny, thick, slightly yellow layer is formed. No liquefaction of media or growth into media or along stabs occurred. In bouillon, after a week, there is slight cloudiness, but no surface growth. Most of the growth in liquid media occurs as a fine white deposit at the bottom of the tube. In bouillon cultures a white growth along the glass above the surface of the media occurs in about a week.

Microscopical Examination.—The cultures show round cells from 1 to 6μ in diameter, the average size being 3 to 4μ.

Buds form from large cells or from the smaller cells, but most often from the medium sized cells. The buds measure usually about 1 to 1.5μ in diameter. They may attain a diameter of 2.5μ before separation. Sometimes the bud reaches nearly the diameter of the parent cell.

As the bud begins to separate a deposit of gelatinous substance forms about the neck of the bud, and after separation occurs it appears as a thick line at the area of separation. The substance stains blue with methylene blue, a dark purple with Wright's stain, and a red color with Giemsa's stain or fuchsin. When cells occur in masses, this substance forms similar lines between adjacent cells, and the cells often are flattened to form hexagons. If the cells become separated the material is drawn out into fine threads connecting the cells, similar to those in the cases. The torula tends to occur in masses in cultures, probably on account of this secretion. When growing in hanging drops it forms close groups.

The medium sized torula cell has a definite wall 0.5 to 1μ thick, staining easily with methylene blue or with Gram's stain. The cell interior, as a rule, cannot be made out to have a definite nucleus. Examination of the organism from cultures is much less satisfactory than in sections of animal lesions. In smears from young cultures a stain with methylene blue shows one to six small, very dark staining masses, irregularly distributed in the cell. In old organisms these are not present. The whole interior of the cells in young cultures usually takes a deep blue stain, in which one or more lighter staining areas may be apparent. Rarely the interior appears coarsely granular. In older cultures, especially in the larger cells, the interior does

not stain so uniformly. There is a darker staining mass in a light staining cell. The mass sometimes appears like a nucleus, and occasionally is surrounded by a thin line like a membrane and may contain a vacuole. In some cases the vacuole appears to have distended the mass, so that the dark staining material lies next to the cell wall. Similar appearances are seen in the animal lesions. With Wright's stain a definite darker staining mass one-third to one-half the diameter of the cell is usually present, eccentrically situated, in the smallest organisms. In the larger ones the mass becomes more irregular in outline and position. In the process of budding part of the chromatic material flows out into the bud and is separated by the constriction of the bud. No nucleolus can be made out.

Torulæ are widely distributed in nature. They occur in the earth, on trees and fruits, in wasps' and bees' nests, and on these insects. Classification of different varieties has been attempted, but is unsatisfactory, on account of the slight and inconstant differences. Weis, in an investigation of four torulæ, found great similarity. None of them fermented sugars. The principal differences were as to the presence or absence of close or open budding, whether they formed top or bottom growth in liquid media, the presence of growth on the glass of the tube, and the amount of gelatinous secretion. These characteristics varied greatly until the organisms had been under cultivation for months. Our torula shows close budding, bottom growth with no growth on the surface, yeast ring after a week's growth, and distinct gelatinous secretion.

VI. TABLE OF DIFFERENTIAL POINTS BETWEEN TORULA INFECTION, OIDIOMYCOSIS, AND COCCIDIOIDAL GRANULOMA.

Torula infection.	Oidiomycosis (usually termed blastomycosis).	Coccidioidal granuloma.
	Cell wall.	
Stains diffusely and easily with methylene blue and hematoxylin.	Does not stain, as a rule, with methylene blue or hematoxylin. Occasionally in deeply stained preparations it partially takes a faint color.	Stains faintly with hematoxylin and methylene blue, but not diffusely; the inner and outer layers are principally stained.

TABLE OF DIFFERENTIAL POINTS—*Continued*.

Torula infection.	Oidiomycosis (usually termed blastomycosis).	Coccidioidal granuloma.
Protoplasm.		
In large forms not evident. In medium sized forms often shrunken into an irregular shape, and takes a diffuse red stain with few differentiated droplets. In small forms fills the organism and has dark staining masses.	Usually slightly shrunken regularly from the wall. Usually stains well and appears uniformly granular, with occasional vacuoles or droplets. It colors a reddish blue with eosin and methylene blue.	Adheres to the capsule, often forming a rim inside it. It is greatly variegated with all the changes attendant on the process of endosporulation. Stains well as a rule.
Size.		
1 to 13μ.	3 to 4μ to 20μ.	5 to 85μ.
Reproduction.		
By budding, often with the production of small organisms which can bud before enlarging.	By budding; usually the bud is $\frac{1}{2}$ to $\frac{3}{4}$ the diameter of the parent cell and grows to the original size before it reproduces.	Never buds. (Buds may be simulated by cells in apposition.) Produces ascospores.
Occurrence.		
Always with a clear zone about it composed of gelatinous material.	No clear zone except in rare cases in the bone marrow.	No clear zone.
Cell products.		
A gelatinous material which often fills the lesion.	No gelatinous material.	No gelatinous material.
Action on tissues.		
Destroys the cells it is enclosed in and appears in large vacuoles which are probably due to destruction of the cell protoplasm.	Distends the cells it is enclosed in, but does not occur in large vacuoles.	Distends the cells it is enclosed in, is closely surrounded by the cell protoplasm, and often the cell shows no evidence of injury.

TABLE OF DIFFERENTIAL POINTS—*Continued*.

Torula infection.	Oidiomycosis (usually termed blastomycosis).	Coccidioidal granuloma.
Character of lesions.		
1. Lesions with solution of tissue, filling with gelatinous material, slight chronic reaction.	No such lesions.	No such lesions.
2. Nodules with or without caseation composed of epithelioid cells, giant cells, and lymphoid cells. Caseation occurs centrally and apparently depends on the action of the cell products.	Nodules with or without caseation. Caseation is secondary to a crowding of cells, apparently.	Nodules composed of epithelioid cells with occasional giant cells. Caseation is secondary to large accumulations of cells and is peripheral.
No collections of polynuclear cells.	Abscesses deep and superficial.	Abscesses deep and superficial.
No cutaneous lesions.	Miliary epidermoid abscesses.	Cutaneous ulcerations.
Does not attract polynuclear leukocytes in any stage.	Usually attracts polynuclear leukocytes.	The smaller forms especially attract polynuclear leukocytes.
Clinical course in man.		
A chronic disease of the nervous system, without constant or great fever or leukocytosis.	A chronic skin disease or general infection with fever and leukocytosis.	A chronic skin disease or a general or brain infection with fever and leukocytosis.
Organs affected.		
Brain and meninges, lungs, liver, spleen, kidneys. Not skin or bones.	All organs, always the skin, often the bones.	All organs, often the skin.
Reaction to treatment.		
Not helped by salvarsan. No data as to effect of iodides.	Usually helped by iodides.	Not helped by iodides.

TABLE OF DIFFERENTIAL POINTS—*Concluded*.

Torula infection.	Oidiomycosis (usually termed blastomycosis).	Coccidioidal granuloma.
Pathogenicity in animals.		
Marked for mice and rats, slight for guinea pigs, rabbits, and dogs, where brain lesions are the principal ones.	Slight or absent for all animals tried.	Marked for all animals tried.
Organs affected in intraperitoneal inoculation.		
Brain, lungs, liver, spleen, kidneys.	Peritoneum, occasionally lungs. Experiments reported in which miliary abscesses or nodules were produced in various organs by intravenous inoculation. None mentioned in the brain.	Lungs, spleen, liver, kidneys, peritoneum.
Organism in culture.		
Reproduces by budding. Never produces mycelium.	Always grows mycelium sooner or later.	Always grows mycelium and aerial hyphæ.

VII. DISCUSSION.

Historically there is great interest in the fact that torulæ were proved years ago by several observers to have marked pathogenic action in animals, with frequent production of brain lesions. It was also shown that torulæ are widespread organisms in nature. It would not have been too venturesome to have prophesied that the organisms would be found at some future date producing nervous disease in man. We have found that cases do occur. It will take time to determine how common this type of infection is, but many facts indicate the possibility of its being frequently present without recognition. A number of factors work to conceal its nature. The organism is not very pathogenic and the lesions are recoverable. Thus, many infections may occur during life without causing noticeable symptoms. At autopsy lesions may be found, but, if in the healed stage, the organisms will be practically or absolutely indis-

tinguishable, and the picture that of a slightly atypical tuberculosis, caseation being slight or absent.   In animals we frequently produced lesions in nearly all organs, some of which were indistinguishable from miliary tubercles without caseation, or with slight central necrosis.   In our human cases the slight lesions outside of the nervous system produced no symptoms in the first case; in the second case, with extensive lung lesions, only an indefinite history of lung symptoms is given.   The lesions closely resembled tuberculosis, and in them the organisms had practically disappeared.   Without brain examination we could not have recognized the nature of the cases.   In the literature are many cases of nodular lesions in internal organs which do not seem to be tuberculous in origin, although the lesions resemble tubercles.   Wolbach reports several, and mentions peculiar crystalloid bodies in the giant cells, which we found in our second case.   Other cases in our recent hospital records are of the same character.   Thus the tendency toward recovery, the often slight involvement of organs, the destruction of the parasite, and the resemblance to tuberculosis, render it possible that the infection is common, but not recognized.

The probability of its recognition in brain examinations is greater, but even here there are several sources of error.   The gliosis and thickened meninges, with slight atrophy of the convolutions, the chronic character of the meningitis, with many plasma cells, and often hyaline organisms, make confusion with syphilis easy.   When the lesions have progressed to caseation, the diagnosis of tuberculosis might be made.   There is no identity on careful examination; the caseation is not typical, its character and distribution are different, and only careful microscopical examination shows the organisms.   The perivascular lesions are often small and would escape discovery unless the brain were completely sectioned.   Many cases would easily escape recognition in the often careless routine brain examination.

The factors which will aid the recognition of torula infection are: discovery of early lesions in internal organs before the parasite is destroyed; realization of the finer points of difference from tuberculosis; complete brain examination; cultures, especially on carbohydrate media at room temperature; and injections of fluids and cultures into white rats intraperitoneally, with microscopical examination, especially of the brain.

Torula infection is essentially a chronic disease. In man the symptoms are measured by months; in animals the lesions last weeks or months. Its total duration as a general disease cannot be determined in the human cases, for the first organ lesions probably produce no symptoms. As a nervous disease it is clinically essentially chronic, and the pathological reaction is always one of extreme chronicity.

The lesions in internal organs, outside of the nervous system, are recoverable. In the human cases and in nearly all the animal cases there is evident a strong tendency toward destruction of the parasites, with the production of small nodules or large masses of connective tissue. In our animal experiments we have seen the various stages of this conversion. In one animal, however, which died from the infection after 5 weeks, there was no tendency toward recovery in the lung lesion, but some in the other lesions. Rusk's case and Frothingham's case showed similar lung lesions. In the brain the tendency toward recovery is not so marked. Many of the lesions in the human and animal cases, especially the intracerebral lesions, show little evidence of defense by cell proliferation or destruction of the organisms. In other instances there is marked phagocytosis by invading mononuclear cells with partial disappearance of the organisms; and in some animals all the brain lesions are seen in a healing stage. The meninges have a more effective defense than the brain.

As to localization of lesions and organ resistance, the animal experiments show a marked tendency to the production of a general infection from intraperitoneal inoculations, with especial involvement of the nervous system. Lesions were found in the brain, meninges, lungs, spleen, liver, kidneys, and lymph nodes. The liver showed the least extensive lesions, the spleen more involvement, and the lung, kidney, and brain the greatest involvement. Sometimes there was more involvement of the brain than of any other organ. This was the case especially in the higher animals. In the dog there was only one small lesion in the kidney; there were none in the other organs, but many small nodules in the brain. The rabbit had one liver lesion, but no other lesion except a meningitis of considerable extent. The mouse inoculated intraperitoneally with the ventricular fluid had only a meningeal lesion; a mouse inoculated with the torula

culture had only a peritoneal lesion, besides the meningitis. In all rats there was a general involvement of many organs, but the brain lesions in the later stages showed more evidence of activity than those of other organs. The same distribution is evident in the human cases; in our cases the organ lesions, as a rule, were insignificant with the exception of the lung lesions of the second case. Histological examination shows a greater destruction of the organisms in the spleen, kidney, and liver than in the lung, and in the lung greater than in the brain. The brain, however, has some power of resistance and recovery. The results all show the peculiar low resistance of the nervous system to torula infection. In higher animals where the resistance is more marked the difference between the lesions in and outside of the nervous system becomes more striking, for here the nervous lesions are the only ones which form or progress in any degree.

The tendency toward the production of brain lesions is in sharp contrast to the action of the other organisms studied, where intraperitoneal inoculation produced brain lesions in no case. Our experiments again support the differences in the human cases, and show that organ resistance in man is not different from that in animals.

The localization of the lesions in the nervous system is in the meninges, in the perivascular spaces, and in the brain substance by extension from these lesions. In the human cases and in animal experiments lesions were also found in the choroid plexus, in one human case in the aqueduct of Sylvius, and in another about the foramen of Magendie. Lesions in the basal ganglia, internal capsule, frontal lobes, and cerebellum occurred. A perineuritis was seen in one case. This tendency toward localization in critical points obviously makes the production of even small lesions of great importance. In animals the same distribution is evident, involvement of the cerebellum being especially noticeable.

Resulting from the chronicity and localization of the lesions are the symptoms in man. Fever was evident only at the onset in the cases and was moderate in degree, the white count was low in one case when fever was present and in others at various periods. We have already discussed the spinal fluid findings in the cases. The variability of the localization will evidently cause no constant symptoma-

tology. The tendency to involve the cerebrospinal fluid pathways is of extreme importance as a method of production of internal hydrocephalus which will cause confusion with cerebral tumor, especially in connection with the localizing symptoms of the other lesions. The optic nerve may become involved, or choked disc may be secondary to other lesions. The frequent occurrence of psychic symptoms is in accord with the tendency toward involvement of the frontal lobes and basal ganglia.

With the exception of syphilis there is little accurate knowledge of recoverable chronic infections of the central nervous system. There is clinical evidence in the reports of pseudotumors and other conditions that such cases may not be uncommon, and the torula infection may have importance in this regard.

The Mechanism of the Disease.—The mode of infection in the human cases is uncertain. Of especial interest is the lesion in the esophagus in Türck's case, from which organisms were isolated similar to those in the spinal fluid. However, yeast-like organisms are common in the throat, and a similarity in cultures cannot be taken to mean identity. In our second case a sticky pharyngeal exudate was noted, but no examination made. In Rusk's case and in our second case there was an evident possibility of origin in an infection of the lung. On the whole, the respiratory tract, as in oidiomycosis, seems the most probable atrium. It is possible that infection may occur together with a tubercular infection. Ascending infection through the fallopian tubes is a possibility.

The path of distribution after infection occurs, in many cases at least, is through the lymphatics to the blood stream. Our second case, with the numerous lesions in the bronchial lymph nodes and the miliary lesions in the spleen and liver, suggests this mode of spread. In the animal experiments transport by lymphatics is plain. Lesions in the retroperitoneal nodes after intraperitoneal inoculation were marked. In an intrathoracic injection, mediastinal nodes were involved. The miliary lesions in all organs are due to blood infection.

In all lesions engulfment of organisms by giant and large mononuclear cells was a marked feature. The organisms continue to live and multiply within these cells and after·destroying them are set free. Thus the methods of defense on the part of the host may spread the infection.

In the brain lesions additional methods of spreading are evident. Organisms are carried along the perivascular spaces of the vessels and start new intracerebral lesions. Or there is direct extension by solution of tissue. This extension by methods similar to those of the ameba is not so evident in other organs, probably because the resistance is so much greater that the organisms are quickly destroyed. It did occur, however, in the lung of an animal with low resistance. Nerve lesions are started by organisms carried into the sheath by the fluid currents.

In the study of the relationship of the organism to its host histology furnishes evidence of a method of attack by mechanical means, the pressure of the growing mass of parasites, and by chemical means, the destruction of the cells of the tissues. The latter factor is the more important. Another means of offense is a weakening of the host by the accumulation of its defensive cells in critical spots so as to interfere with function. In this mode of attack the torula has the great advantage of being able to produce lesions easily in the nervous system, and in almost any part of the nervous system. In our cases it was evident that a great deal of the disease picture was produced by the interference with the passage of cerebrospinal fluid by focal lesions; and the weakening of the host produced by the internal hydrocephalus gave the organism a chance to multiply with greater freedom.

In the older lesions the organism becomes fainter staining, or collapsed, and finally disappears. In some cases only miliary lesions exist. Old cultures of torula do not show disappearance of the organisms, so that the destruction in the lesions is probably by the action of the tissues. The destruction occurs in cells or out of cells. In some cases, however, it is not evident. No production of agglutinins was found in any case. A further advantage possessed by the torula is the production of the large thick walled resting cells. These are typical of torulæ in nature, which are highly resistant and long enduring. Dried up cultures of torula several years old immediately grow with rapidity when transplanted, and are pathogenic. In one rat the histological study of the meninges showed only the large, almost hyaline forms, but a pure culture was obtained with ease. It is probable that the infection can remain dormant for a long time, and then start up afresh. We have direct evidence of this

in our second case, where the lung, lymph node, and liver lesions were old, the lung lesions consisting of hyaline connective tissue masses, with only a few thick walled forms, while the brain showed a very active process with young forms predominant. The cerebellar lesion in our first case was an old lesion, consisting almost entirely of the resting cell forms. Here the struggle between the parasite and the tissues had apparently come to a standstill.

The Mode of Multiplication of the Organism.—The small, actively budding organisms found under favorable conditions of nutrition, and the resistant spheres under unfavorable circumstances adapt the parasite to most various conditions.

The histology of the lesions has been fully described, but the relationship of the two principal types has not been explained. The experiments give a plain solution of the question, and show part of the mechanism of the lesions. Possibly the lesion starts by the passage of a small torula into the tissue while in a phagocytic cell from the blood stream. It multiplies within the cell and destroys the cell, and then the organisms are liberated. A single organism in an endothelial cell or a few small organisms in a giant cell have been found as the early lesions. The organism then multiplies and pro- duces a gelatinous material which surrounds it. Phagocytic mono- nuclear cells gather and take up organisms, which multiply within them, destroy the cell, and are set free. Giant cells form and undergo a similar process. The surrounding tissue meanwhile is partly pushed apart, partly dissolved. Strands of connective tissue often form about the gelatinous capsule, giving rise to a honeycombed appearance, the spaces each containing an organism. This is a stage common to all types of lesion. The varying degrees of resistance of the animal determine the further fate of the lesion. In a non-resistant animal or organ, the ameba-like extension by solution of tissue occurs, with a feeble defense by the production of mononuclear cells and slight connective tissue. This type of lesion occurs especially in the brain substance, but is seen in the lung and to a less extent in other organs. It is a striking and characteristic lesion.

If the animal is comparatively resistant, there is more cell accumu- lation and proliferation, with some destruction of the organisms. Epithelioid and lymphoid cells gather, the central part of the lesion undergoes necrosis and partial caseation, in which at first the organisms

can be seen in honeycombed material, and later almost disappear. In lymph nodes this type of lesion is most extensive and furnishes a resemblance to a scrofulous gland.   If the destruction occurs promptly the lesion is small and nodular, with slight central necrosis or caseation or none, and is like a miliary tubercle.   Organisms may or may not be recognizable.   Lesions thus were found in all organs.   If infection occurs simultaneously, in many adjoining foci, there is a diffuse chronic inflammatory tissue consisting of giant cells, lymphoid, epithelial, endothelial, and plasma cells, with connective tissue formation, and torulæ enclosed in small spaces.   Such lesions are especially frequent in the meninges and correspond to diffuse tuberculous tissue.   Early and slight diffuse lesions in the meninges and brain call out only fluid and large phagocytic mononuclear cells. When the organisms are overcome quickly, there is slight production of gelatinous material, but gelatinous encapsulation is always plain. Very old lesions become converted into fibrous tissue masses.

On account of the histolytic action so striking and characteristic in early lesions and in brain lesions, it is suggested that the organism be called *Torula histolytica*.

We wish to emphasize the fact that not only do the lesions resemble those of tuberculosis, but they resemble them more closely than do the lesions of any other disease.   Coccidioidal granuloma, considered by some authors to produce lesions indistinguishable from tuberculosis, in our experiments showed a marked tendency toward miliary or larger accumulations of polymorphonuclear leukocytes, especially around sporulating organisms.   Caseation was at the periphery of large collections of polynuclear and epithelioid cells and did not occur in the small epithelioid cell masses.   The parasites were large and easily seen.   Oidiomycosis bears still less resemblance to tuberculosis.   In torula infection the parasites are often hyalinized and, being about the size of tissue cells, are almost impossible of recognition in advanced lesions.

VIII. SUMMARY.

One of the problems in our work was the relationship of the organisms causing the diseases termed blastomycoses.   We have shown the confusion existing in text-books, where the various diseases are

described as one disease, or as different manifestations of the action of a single organism in different states. The study of the literature convinced us that coccidioidal granuloma was a disease distinct clinically, pathologically, and biologically from other diseases called blastomycosis. The work of Wolbach, and MacNeal and Taylor, who described the life cycle of the organism of dermatitis coccidioides, never present in the other cases, makes the biological distinction evident; the summaries of Ophüls, Ryfkogel, Hektoen, and Brown and Cummins, establish the clinical differences. We have not regarded it necessary to repeat these summaries. Our experiments bear out the former work. The lesions produced by *Coccidioides immitis* are not to be easily confused with those of blastomycosis, or torula infection. For the details of the differences between the diseases we refer to the discussion in the review of literature and to the table. We lay emphasis on the point, not because we do not consider that other workers have not made it sufficiently clear, but because it is not yet recognized in text-books, and because our experiments bear out the former statements.

Having seen that coccidioidal granuloma was a distinct disease, we turned our attention to the blastomycoses. We found in the literature two cases of skin and general infection produced by a true yeast, with endospores in culture. Both cases were observed by Buschke, and appeared to be distinct from the American cutaneous disease. Frothingham's discovery of torula infection in a horse indicated another type, but no such cases had been reported in human beings.

Our cases were distinct from the larger part of the reported cases of blastomycosis in their clinical histories and pathology. It did not seem improbable that in the early study of blastomycosis such cases had been described, but their nature not recognized. We studied the original reports of all the cases of systemic blastomycosis and found that nearly all the cases were similar to each other as far as we could tell from the printed reports, except those involving the brain. Among these there were obvious differences. First there were six cases like the other systemic cases, but in which the brain became involved as part of the general infection, which always included skin manifestations and often bone lesions. The symptomatology was not

perceptibly influenced by the brain lesions. The pathology of the brain lesions resembled that of the other lesions. Different from these were four cases in which there were no skin lesions, but in which a general infection occurred with brain lesions which caused the predominating symptoms. Pathologically the lesions were distinct in many ways; but principally in the extension by solution of tissue, the always chronic reaction, and the production of a gelatinous material in the lesions.

Our first case was evidently identical with this latter group. Our second case was not fully identifiable by the study of the literature alone, for the peculiar intracerebral lesions were not present, and the parasite occurred in greater numbers of small forms. Such forms occurred in the meninges of the first case, but not in the intracerebral lesions, and were not described in the literature. In the experimental meningitis in a mouse produced by the injection of a culture of the ventricular fluid from the second case, however, large organisms were produced, identical with those of the first case, and intracerebral lesions of the same type were seen in process of formation. Thus our two cases were proved to be alike in origin.

Frothingham's case of torula infection was evidently the type of infection of these cases. In our animal experiments with torula we found both forms of parasites present in the lesions in varying proportions according to the extent and activity of the process. In a very active lesion enormous numbers of small organisms similar to those of the second case occurred; these were seen especially in the meningeal lesions. In older lesions, tending toward recovery, or in those slowly progressing, and in the higher animals, the larger forms predominated. In sections of the original horse lesions, small forms are entirely absent. Our experiments resulted in the production of all the variations in lesions and organisms seen in the cases. The animal experiments thus provided the necessary steps for the clear correlation of all the human cases as cases of torula infection.[1]

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[1] Doubt has occasionally been thrown on the possibility of distinguishing a true yeast or a torula from an oidium or a degeneration form of some higher fungus by mere examination of the tissues. In the case of certain organisms which do not produce especially characteristic pathological effects, and are sufficiently similar morphologically, this may be true, but in the case of the three organisms

For all points of difference in the types of disease studied, we refer to the summary on page 60 and to the table on page 79.

As we have shown (page 60), the term blastomycosis has resulted in confusion of different diseases in the past and will continue to do so if used in the future, on account of its insufficient biological significance. Oidiomycosis is the proper name for the diseases occurring near Chicago and caused by the organisms budding in tissues and producing mycelium in cultures. Torula infection, coccidioidal granuloma, and yeast infection sufficiently designate the other diseases studied.

We wish to express our thanks to Dr. W. T. Councilman, as the instigator and constant helper and advisor in the work; to Dr. Harvey Cushing, for permission to use the cases; to Dr. S. B. Wolbach, for the cultures of the organisms and for many useful suggestions; to Dr. L. Frothingham, for the use of slides and tissue from his original case; to Dr. F. B. Mallory, for the use of slides; and to Dr. Roland T. Thaxter, for his careful examination of the organisms.

we studied there were no lesions where confusion as to the cause could exist after careful examination. Each variety of organism had a characteristic appearance in the tissues. When the histology and the distribution of the lesions are also considered, enough factors exist to make full identification possible. Lesions (not described in this paper) were also produced with various oidia (*Oidium albicans* and others, isolated from human throat infections), and no appearance was seen which would be confused with any of our other lesions. Reported cases of higher fungus infection in which budding forms occur in tissues are distinct from our cases. If we had had no cultures from the cases and no culture of torula from other sources for experimental purposes the nature of the organisms could not have been stated positively. In future other forms of torulæ or yeasts may be found which are not sufficiently characteristic in their effects to be distinguished from budding forms of higher fungi by histological examination alone. The cases described in this paper are probably a small part of those existing in nature and not yet studied. Thus, Escomel's recently reported cases of surface infection with budding organisms occurring in Peru and Bolivia are apparently caused by another variety of either yeast or torula. No mycelium is ever produced; the presence or absence of ascospores is not noted, and no plates are given or details of histology. The classification in this paper does not pretend to be complete, but merely to prevent confusion and to make clear the distinctions possible at the present time.

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X. EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Slight atrophy and pitting of convolutions of the frontal lobe of the first case.

FIG. 2. Lesion in the frontal lobe of the first case.

FIG. 3. Cerebellar lesion in the first case.

PLATE 2.

FIG. 4. Periphery of cerebellar lesion in the first case, showing the method of extension by solution of tissue. Stained with eosin and methylene blue. × 150.

FIG. 5. A group of intracerebral perivascular lesions in the first case. Stained with eosin and methylene blue.

FIG. 6. Single perivascular lesion in the first case. Stained with eosin and methylene blue. × 150.

PLATE 3.

FIG. 7. Small intracerebral lesion in the first case. Apparently the organisms arose from the large organism in the center. The drawing out of the secretion into threads is evident. Stained with eosin and methylene blue. × 2,000.

FIG. 8. Peripheral gliosis in the first case. Stained with phosphotungstic acid hematoxylin. × 1,000.

FIG. 9. Organisms in giant cells in the meninges in the first case. Cresyl blue stain. × 850.

PLATE 4.

FIG. 10. Granulation tissue filling the aqueduct of Sylvius in the first case. × 60.

FIG. 11. Area of meninges in the first case where the pseudotuberculous process is marked. Stained with eosin and methylene blue. × 100.

FIG. 12. Cerebellar lesions in the second case.

PLATE 5.

FIG. 13. Edge of cerebellar cortex showing meningitis in the second case. Stained with eosin and methylene blue. × 100.

FIG. 14. Organisms in meninges in the second case. Note the budding organism. Oil immersion. Stained with eosin and methylene blue. × 1,200.

FIG. 15. Lymph node in the second case. At the upper right hand corner is the edge of a mass of dense connective tissue. Stained with eosin and methylene blue. × 100.

FIG. 16. Lung lesion in the second case. Stained with eosin and methylene blue. × 100.

FIG. 17. Lesion in the internal capsule in the second case. Stained with hematoxylin and eosin. × 50.

FIG. 18. At the left side is the necrotic center of the lesion in Fig. 5. Oil immersion. Stained with hematoxylin and eosin. × 1,200.

PLATE 6.

FIG. 19. Giant cell containing organisms in the meninges in the second case.

FIG. 20. Organisms in a large vacuole in a meningeal giant cell of the second case.

FIG. 21. Meninges of a mouse infected intraperitoneally with a culture of ventricular fluid of the second case. Stained with hematoxylin and eosin. × 250.

FIG. 22. Radiating threads in an organism from Fig. 3. Oil immersion. Stained with hematoxylin and eosin. × 2,000.

FIG. 23. Radiating threads in organisms in cerebellar lesion in the first case. Cresyl blue stain. × 1,000.

FIG. 24. Original torula infection in the lung of a horse. Stained with hematoxylin and eosin. × 300.

FIG. 25. Intracerebral lesions in a rat infected intraperitoneally with torula.

FIG. 26. Torulæ in experimental lesion. Oil immersion. Stained with eosin and methylene blue. × 1,000.

FIG. 27. Torulæ in an endothelial cell in the lung of a rat. Oil immersion. Stained with eosin and methylene blue. × 2,000.

PLATE 7.

FIG. 28. Torula lesion in a rat infected intraperitoneally. Stage of healing. Stained with eosin and methylene blue. × 150.

FIGS. 29, 30, 31. Torulæ in experimental lesions. Oil immersion. Note the apparent nucleus in Fig. 31. × 2,000.

FIG. 32. Budding torulæ. Note the extremely small buds. Oil immersion. Stained with eosin and methylene blue. × 1,000.

FIG. 33. Variations in the size of torula in an experimental lesion in a rat (lung). Oil immersion. Stained with hematoxylin and eosin. × 2,000.

FIG. 34. Meningeal lesion in a rat infected intraperitoneally with torula. × 2,000.

FIG. 35. Pseudotuberculous lesion in a peritoneal nodule in a rat infected with torula. Note organisms in spaces. Stained with eosin and methylene blue. × 50.

FIG. 36. Lesions like miliary tubercles in the lung of a rat infected with torula by heart injection. Stained with eosin and methylene blue. × 50.

PLATE 8.

FIG. 37. Organisms in a destroyed giant cell in the meninges in the second case. At the upper left hand corner are two red blood corpuscles for comparison of size. Camera lucida drawing. Oil immersion. Mallory's aniline blue connective tissue stain.

FIG. 38. Beginning lesion in a meningeal giant cell in the second case. The organisms are thinner walled than in Fig. 1. Camera lucida drawing. Oil immersion.

FIG. 39. Organisms in the meninges of a mouse infected intraperitoneally with a culture of ventricular fluid of the second case. Note budding of the small organism. Camera lucida drawing. Oil immersion.

PLATE 9.

FIG. 40. Organisms in a giant cell in the lung of a rat infected · with torula. The central part of the cell contains the completely finished drawing. · Camera lucida. Oil immersion.

FIG. 41. Organisms in the lung of a rat infected with torula. Camera lucida. Oil immersion.

FIG. 42. Very small torulæ in the cerebellar cortex of an infected rat. Camera lucida. Oil immersion.

(Stoddard and Cutler: Torula Infection.)

(Stoddard and Cutler: Torula Infection)

PLATE 6.

37

38   39

(Stoddard and Cutler: Torula Infection.)
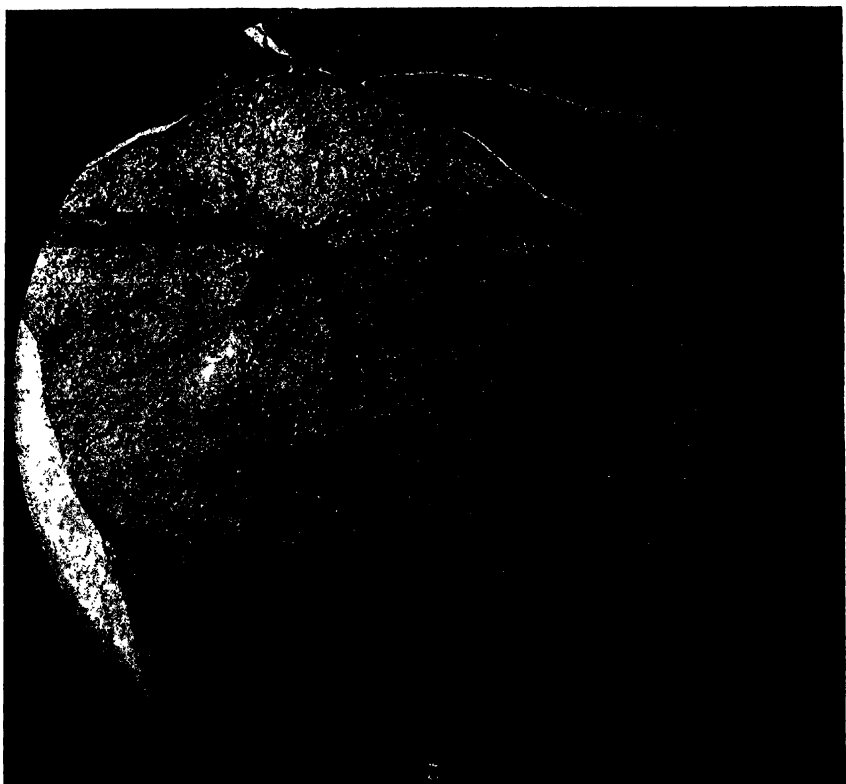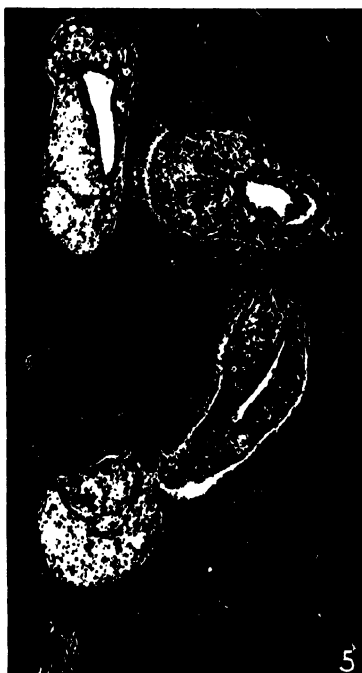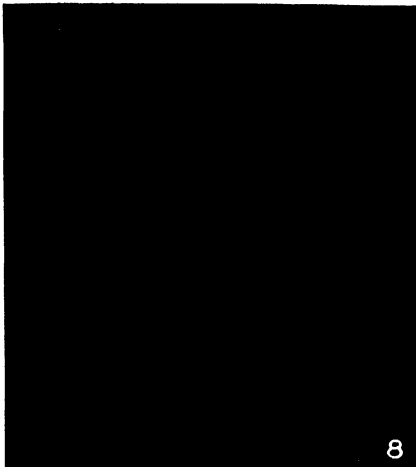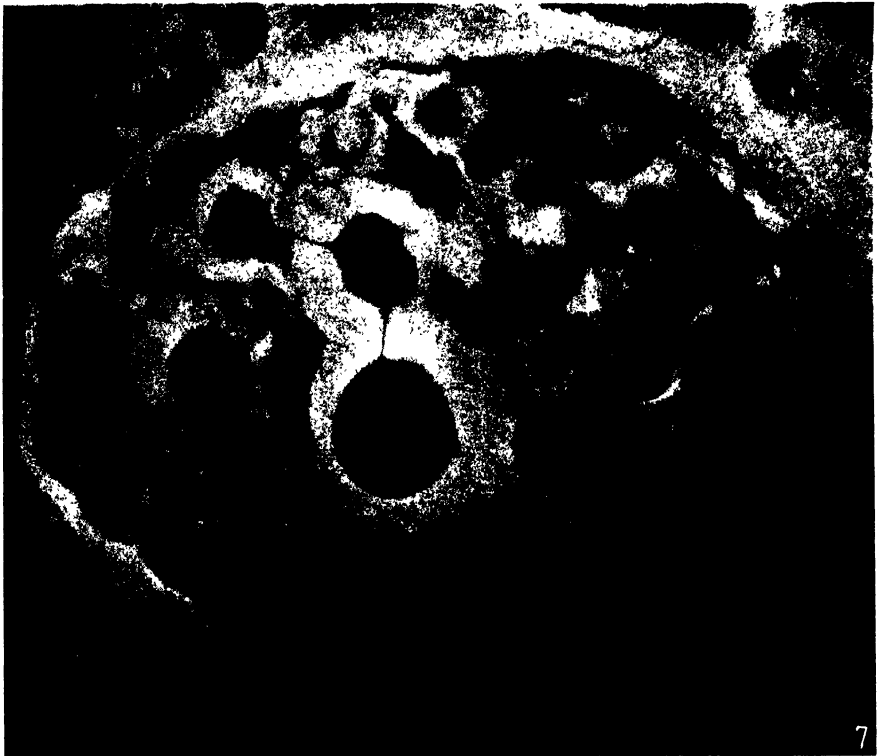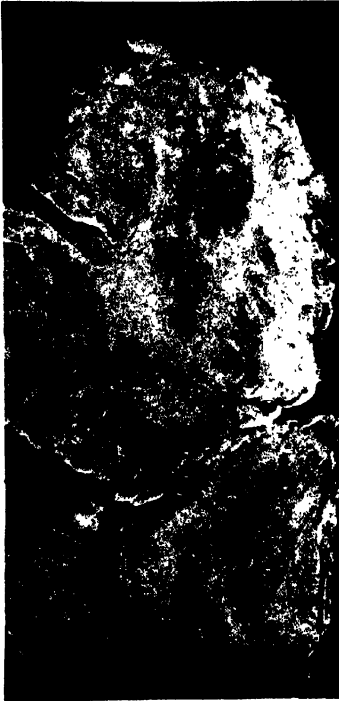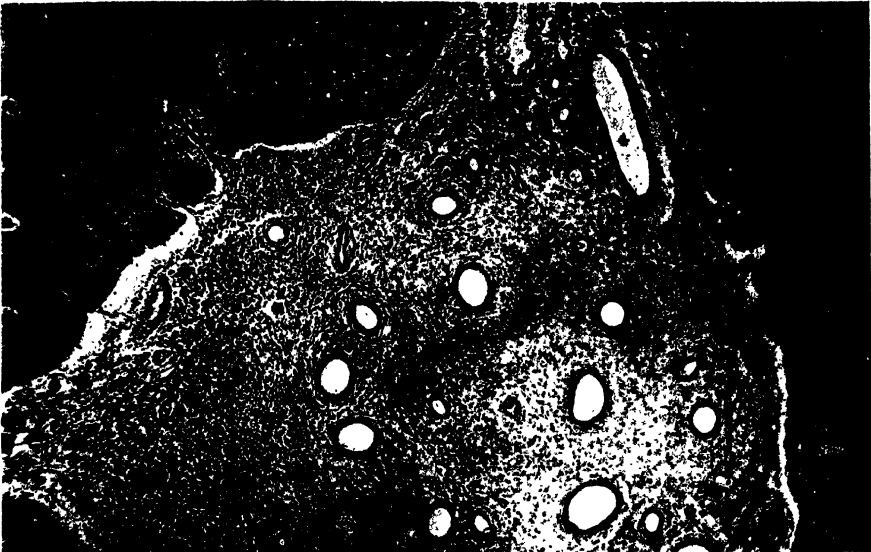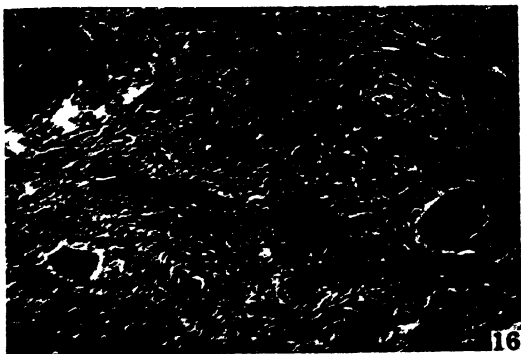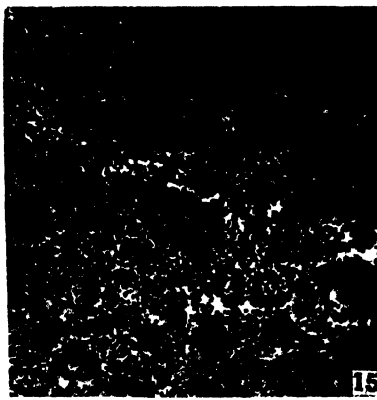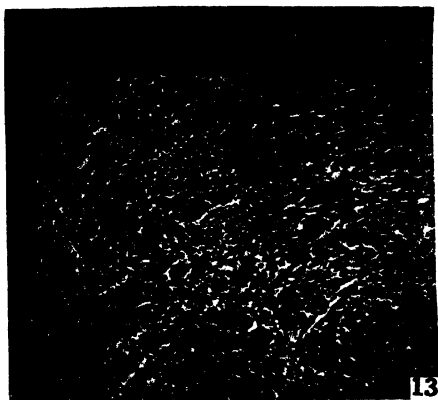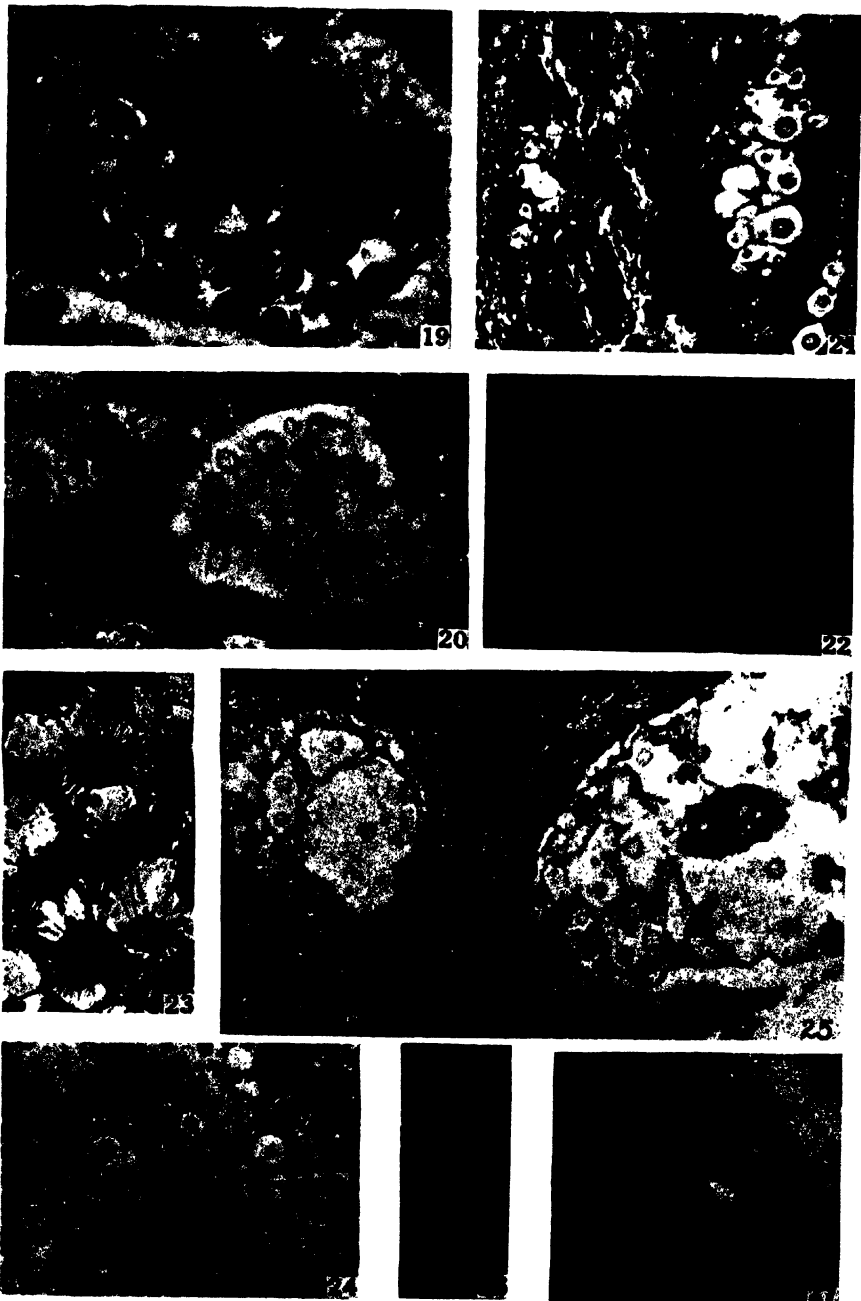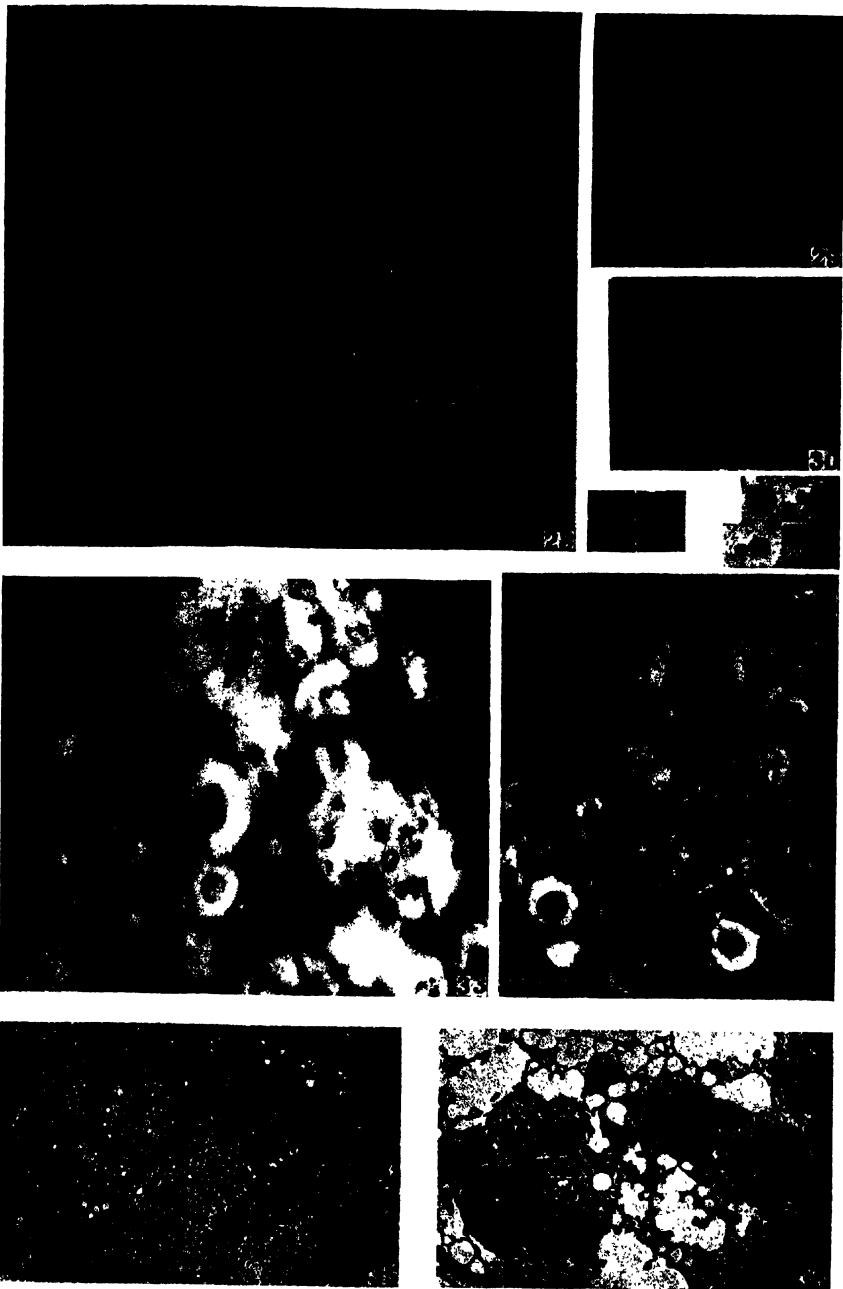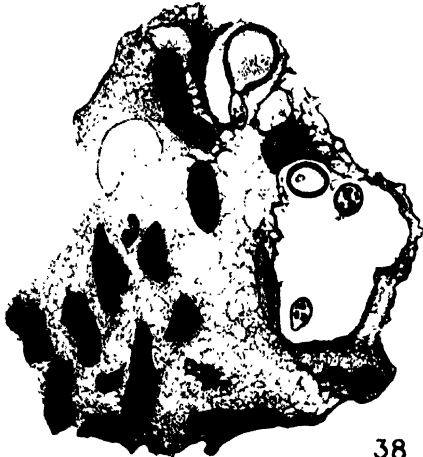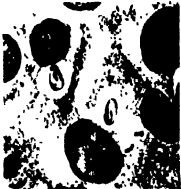
(Stoddard and Cutler: Torula Infection.)

A TRANSPLANTABLE CARCINOMA OF THE GUINEA PIG.

BY F. S. JONES, V. M. D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 29 TO 32.

(Received for publication, September 15, 1915.)

The successful transplantation of rat and mouse tumors has led to many attempts to transfer the tumors of other species. The venereal lymphosarcoma of dogs, transferred by coitus, has been studied by a number of investigators, especially Sticker.[1] Von Dungern and Coca[2] described, and succeeded in transferring an epidemic tumor of the hare. They[3] were able to transfer the venereal dog tumor to foxes and the hare tumor to rabbits. The status of these growths must be considered doubtful since it has been well established that neoplasms in general are only transplantable within the species in which they occur spontaneously. Ribbert[4] has described a fibroma of the dog which he inoculated successfully into two other series of dogs.

Rous[5] succeeded in transferring a spindle-celled sarcoma of a fowl. Later he and his collaborators reported a number of other transmissible avian sarcomata. Fujinami and Inamato[6] have transplanted a myxosarcoma of the fowl. More recently Schultze[7] described a spindle-celled sarcoma of the rabbit which he had propagated through twelve tumor generations. In the laboratories of the Imperial Cancer Research Fund of London another rabbit sarcoma has been studied.

Up to the present a transplantable tumor of the guinea pig has not been reported. Few data are obtainable concerning the tumors of this species; apparently they are rare. Sternberg[8] reported a metastasizing adenocarcinoma of the mammary gland of the guinea pig. He did not attempt transplantation.

[1] Sticker, A., *Ztschr. f. Krebsforsch.*, 1904, i, 413.

[2] von Dungern and Coca, *Ztschr. f. Immunitätsforsch., Orig.*, 1909, ii, 391.

[3] von Dungern, *München. med. Wchnschr.*, 1912, lix, 238.

[4] Ribbert, H., *Centralbl. f. allg. Path. u. path. Anat.*, 1910, xxi, 625.

[5] Rous, P., *Jour. Exper. Med.*, 1910, xii, 696.

[6] Fujinami, A., and Inamato, K., *Verhandl. d. jap. path. Gesellsch.*, 1911, i, 114.

[7] Schultze, W. H., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, xvi, 358.

[8] Sternberg, C., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, xvi, 362.

The tumor which I have transplanted occurred in the mammary gland of an old female guinea pig which, judging from the condition of the breast, had recently suckled offspring. This animal was removed from the Institute breeding pens, with a number of others suffering with abscesses. It had a large fluctuating abscess in the submental region and a large, rather firm, sharply circumscribed tumor in the left mammary gland. This tumor lay in the substance of the gland directly beneath the skin, but was not attached to the subcutaneous tissue or musculature. It was hemispherical in shape, of a grayish white color, and measured 4.5 by 4 by 3.6 cm. The mass was enveloped in a delicate, translucent capsule. It was removed and cut across. Just beneath the capsule was a zone of grayish white, translucent tissue 0.5 to 1 cm. wide. A milky fluid exuded from its cut surface. Toward the center of the tumor this zone gradually faded off into a rather granular necrotic material. The color of this portion of the growth varied from yellowish white to ocher. The diagnosis of cancer was made from frozen sections of the outer zone of grayish white tissue. Examinations of the regional lymph glands and other organs for metastases were negative.

Bits of the peripheral layer of the tumor were inoculated by means of a trocar into the subcutaneous tissue of the groin and into the muscles of the upper leg of sixteen young guinea pigs. Most of the animals were between two and three weeks old. Animals of this age were chosen because it has been frequently shown that young individuals are especially favorable hosts for tumor grafts.

First Tumor Generation.

Oct. 30, 1914. Sixteen young guinea pigs were inoculated with two small pieces of the tumor in the subcutaneous tissue of the left groin and the muscles of the left upper leg. The pieces varied from 0.15 to 0.2 cm. in diameter.

On Jan. 15, 1915, one of the animals showed a swelling in the muscles of the leg at the site of inoculation (Fig. 1). The growth was irregularly rounded and protruded sharply from the surface of the leg. It measured 2.1 by 1.7 by 1.2 cm. The subcutaneous graft in the left groin could not be detected on palpation.

On Jan. 20, 1915, the animal was anesthetized with ether and the major portion of the tumor removed. It lay within the muscles, protruding sharply from the general contour of the leg. Externally it was covered with a thin sheet of muscle. The mass was encapsulated on its external surface with a thin, trans-

lucent layer of connective tissue. The inner surfaces were firmly fixed to the muscles. Nearly all of the tumor was removed in small pieces. These consisted of pink, friable tissue, some portions of which were hemorrhagic. A few circumscribed areas of necrosis were noted. The incision in the skin was closed with silk. Most of the tissue was used to inoculate another series of animals. The remainder was fixed in Zenker's fluid for microscopic study.

The other animals of this series were under observation for over 6 months. None of them developed tumors.

Histology of the Primary Tumor.

Sections of the original tumor show it to be a carcinoma. The cells are arranged in acini, or more or less irregular clumps, supported by a delicate connective tissue stroma. The proliferating portion is confined to a narrow layer surrounded by a thin capsule. In one section the cancer cells have penetrated this capsule and invaded the mammary gland (Fig. 5). The individual cells in the growing portion are large, with a large vesicular nucleus. They stain well. A few mitotic figures are seen. Toward the center the cells appear shrunken and the nuclei stain intensely, while still further in all cell structure is lost. The tumor is well vascularized, but not hemorrhagic.

A few eosinophils are found along the borders of the tumor. In several places its cells have invaded the fatty tissue of the gland (Fig. 5). The interacinar connective tissue is increased throughout the section. Many of the neighboring acini and ducts show pressure changes. They are flattened and the epithelium is atrophied. The wall of one of the larger ducts adjacent to the tumor has become infiltrated with tumor cells and the growing tissue extends as a considerable mass into the duct lumen. There is a marked collateral hypertrophy of the lining epithelium and the interacinar mass is partly covered by a double layer of non-malignant columnar cells (Fig. 7).

Of great interest are certain changes in the breast that cannot be attributed directly to the tumor. One finds the atrophy, overgrowth of connective tissue, and chronic inflammatory changes seen in the breasts of old animals of other species. Some of the acini at considerable distance from the tumor show proliferative changes. Their epithelium has proliferated until they are nearly occluded with a densely packed mass of cells (Fig. 6). Apparently the cells in lesions

of this type show no tendency to break through the acinar walls.   A few acini reveal still more advanced changes and have almost lost their character.   The remains of such an acinus consist of an irregular clump of cells with an ill defined basement membrane.   As a whole, the changes are similar to the so called precancerous lesions noted by Haaland[9] in the breasts of old female cancerous and noncancerous mice.   McCarty[10] has also described the same conditions of the mammary glands of women suffering from carcinoma of the breast.

The Tumor of the First Transplantation.

It has been stated in the protocol that a tumor developed slowly in one animal inoculated with small pieces of the spontaneous tumor (Fig. 1).   The greater portion was removed, but it began to recur 3 weeks after operation.

Mar. 3, 1915.   The operated tumor remained quiescent for about 3 weeks and then began to grow rapidly.   On this date it measured 2.4 by 1.5 by 1.5 cm.   The enlarged inguinal lymph gland on the same side was felt as an oval disc measuring 1.9 by 1.4 by 0.6 cm. (Fig. 2).   Growth of the subcutaneous graft had not taken place.

Mar. 8, 1915.   The animal was found dead.   The leg tumor measured 2 by 1.6 by 1.2 cm.   It was an irregular flattened sphere with a smooth projecting surface.   The deeper portions had invaded the muscle.   On section the growth was divided into numerous lobules by strands of connective tissue.   Considerable brownish yellow, necrotic material was noted.   The peripheral portions of the tumor were composed of friable, translucent, yellowish white tissue which exuded a milky fluid.

The disc-shaped mass in the inguinal region proved to be a metastasis in which the lymph gland had been replaced almost entirely with tumor tissue.   It measured 1.2 by 1.1 by 0.6 cm. and it was made up of the same type of tissue as the leg tumor.   About 1.5 cm. above the metastasis there were two tiny, discrete, hemispheral, grayish white nodules on the external surface of the abdominal muscle.   These were doubtless a part of the tissue supposed to have been inoculated subcutaneously.

On opening the abdominal cavity a large, irregular, ovoid tumor was found.   It measured 6.6 by 5.0 by 3.8 cm. and lay on the right side between the liver and

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[9] Haaland, M., *Fourth Scientific Report of the Imperial Cancer Research Fund*, 1911, 1.

[10] McCarty, W. C., *Surg., Gynec. and Obst.*, 1913, xvii, 441.

cecum (Fig. 4). Broad bands of connective tissue connected it with the neighboring viscera and peritoneum. Its surface was smooth and glistening. The mass was divided by fissures into four principal lobules which in turn were separated into a large number of small lobulations. The color varied. The sounder portions were grayish white and translucent. Considerable hemorrhage had occurred beneath the capsule, which resulted in a gray and red mottling. The capsule was thin and well vascularized.

On section the surface lobulations were found to extend throughout. The four principal ones were separated by broad bands of connective tissue. At both ends and along the superior border was a layer of translucent, grayish white, nearly homogeneous tissue. The balance of the tumor was practically comprised of pinkish or reddish gray necrotic material.

Other tumors were not found in the abdominal cavity. Gross or macroscopic metastases were not observed in the other organs or glands.

Doubtless the large abdominal tumor was responsible for the death of this animal. From the location and general character of the tumor it seemed evident that it resulted from direct transplantation. Probably the cannula, with which the subcutaneous inoculation had been made, pierced the abdominal wall, and a small portion of the tumor lodged in the mesentery. The inguinal tumor was unquestionably a metastasis from the leg mass to the inguinal lymph gland.

Second Tumor Generation.

Series A.—Jan. 20, 1915. Thirty young guinea pigs were inoculated in the leg muscles and in the subcutaneous tissue of the groin with small pieces of tumor from No. 31.

Feb. 26, 1915. One animal, No. 26, had developed a tiny shot-like nodule in the leg and groin.

Mar. 8, 1915. Animal 26 was found dead. In the muscles of the leg at the site of inoculation were three round, raised, grayish red nodules varying in size from 0.2 to 0.4 cm. In the subcutaneous tissue of the groin 6 small glistening gray nodules were found embedded in the fascia. Microscopically these consisted of living tumor tissue, doubtless that which had been implanted. Metastasis to the other organs had not taken place. Bronchopneumonia caused the animal's death.

Mar. 8, 1915. Two other guinea pigs, Nos. 9 and 38, had developed small tumors in the leg and groin. The greater portion of the leg graft of No. 9 was removed and inoculated into the leg muscles of twenty young animals (3rd Tumor Generation, Series A).

May 1, 1915. The operated tumor recurred very slowly at first, but later grew rapidly. On this date it measured 2 cm. The animal was again anesthetized and a considerable portion of the leg tumor removed; bits of it were injected into twenty-five others (3rd Tumor Generation, Series B).

May 11, 1915. The leg tumor of No. 38 had grown slowly. At this time it measured 1.8 cm. in diameter. The major portion of it was removed, chopped fine, and drawn into a syringe. Five young guinea pigs were inoculated in the leg muscles with small amounts of the hash.

May 15, 1915. The tumor of No. 9 enlarged rapidly after the last operation, but soon began to soften and become a huge, almost spherical swelling. It involved the whole upper leg and hip and extended to the mammary region (Fig. 3). It measured 4.7 by 4.2 by 4.2 cm. In the leg of Guinea Pig 38 a large diffuse wedge-shaped nodule had appeared.

June 18, 1915. Animal 9 was found dead. The leg tumor had begun to ulcerate several days before. At autopsy it was found to be largely necrotic The inguinal lymph glands draining the tumor were enlarged. Gross tumors were not found in other organs. Microscopic examination of the enlarged inguinal lymph glands revealed a metastatic tumor in one of them. Metastatic tumor cells were observed in sections of the kidney. They were scattered in irregular aggregates in the external portion of the medulla. The process seemed to have begun in the interlobular capillaries with consequent invasion and obliteration of the tubules. Metastases were not found in the lungs or other organs.

Series B.—Mar. 8, 1915. Twenty young guinea pigs were inoculated by means of a cannula in the leg muscles and in the peritoneal cavity with bits of the leg and abdominal tumors of No. 31, obtained some hours after death. The animals were under observation over 4 months, but none of them developed a tumor.

Third Tumor Generation.

Series A.—Mar. 30, 1915. Twenty young guinea pigs were inoculated with cannulas in the left leg with bits of tumor from No. 9.

Apr. 16, 1915. No. 103 had a small tumor at the point of inoculation.

May 11, 1915. Three animals, Nos. 103, 77, and 67; had developed tumors of a rather uniform size in the inoculated leg muscles. The largest measured 2.3 cm. and the smallest 1.8 cm. On operation most of the tumor of No. 103 was necrotic. Five young guinea pigs were inoculated in the leg with the better portions of the growth (4th Tumor Generation, Series A). Twenty others were injected in the leg with small pieces of tumor incised from the leg of No. 77 (4th Tumor Generation, Series B).

June 16, 1915. The tumor of No. 67 was incised and several small portions of it were inoculated into the upper leg of 15 young guinea pigs (4th Tumor Generation, Series C).

The Transplanted Tumor.

There has been a gradual increase in the number of takes. In the later generations the tumors have appeared earlier and have grown more rapidly and reached a larger size.

Microscopically the transplanted tumor consists of a zone of variable width of rapidly proliferating epithelial cells surrounded by a thin capsule. The cells may be grouped in acini, but they usually lie in small irregular clumps. Large numbers of them may be undergoing mitotic division (Fig. 9). Degenerative changes similar to those observed in the spontaneous tumor are frequent. At the growing edge small blood vessels are numerous. Occasionally they have the form of thin-walled sinuses (Fig. 8). One is struck by the slight reaction about the tumors; practically no round celled infiltration is observed about their borders. The connective tissue capsule is well defined but thin.

SUMMARY.

An adenocarcinoma of the mammary gland of an old guinea pig has been successfully transplanted through eight successive series of animals. It now appears much earlier and grows more rapidly. The number of takes also has increased. In two instances metastasis to the regional lymph glands (inguinal) has been observed. Once microscopic metastases were found in the kidney. The so called precancerous changes observed in the breasts of women and mice suffering from mammary carcinoma were found in the mammary gland of the spontaneous tumor animal. It is hoped that the tumor may soon be utilized for experimental purposes.

EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. The tumor in the leg of Guinea Pig 31 of the 1st Tumor Generation, 81 days after inoculation.

FIG. 2. Recurrence in the leg of the same animal, at autopsy 47 days after partial removal. Note the metastasis to the inguinal lymph gland.

FIG. 3. Large cystic tumor of the leg of Animal 9 of the 2nd Tumor Generation, 4 months after inoculation. On two different occasions large portions of the growth had been removed.

PLATE 30.

FIG. 4. The abdominal tumor found at autopsy of No. 31 of the 1st Tumor Generation. It has been reflected to show the bands of attachment.

PLATE 31.

FIG. 5.   Cells of the spontaneous mammary tumor invading the fatty tissue of the gland.

FIG. 6.   Epithelial inclusions in the lumen of the mammary acini of the original animal.

FIG. 7.   Growth of the cells of the spontaneous tumor through the wall and into the lumen of one of the larger milk ducts.   The growth of the cancerous epithelium has been accompanied by a collateral growth of the lining columnar cells.   Note the pressure changes of the smaller ducts.

PLATE 32.

FIG. 8.   The characteristic border of growing neoplastic cells with many blood sinuses in a section of transplanted tumor.

FIG. 9.   The transplanted tumor in a later generation showing the usual arrangement of cells.   Many of them are undergoing mitosis.

(Jones: Transplantable Carcinoma of Guinea Pig.)

(Jones: Transplantable Carcinoma of Guinea Pig.)
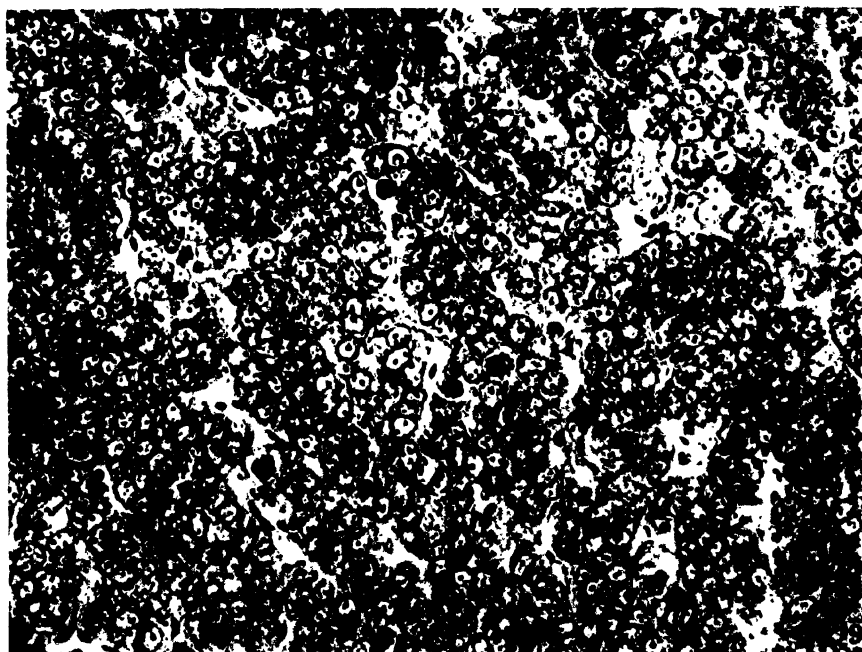
(Jones: Transplantable Carcinoma of Guinea Pig.)

THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

I. METHODS OF PRESERVATION.

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There is practically no mention in the literature of attempts to keep red blood cells alive for a long time *in vitro*. Yet methods for their preservation might have much practical importance, and certainly would possess theoretical interest. Kept cells could be utilized for serum reactions, or for culture media, or even under certain circumstances for transfusion. This last possibility is the more worth considering because of the recent experiments of Abel and his coworkers[1] on plasmaphaeresis. They have demonstrated that the body tolerates well the repeated withdrawal of large amounts of plasma, rapidly placing new fluid in circulation; from which it follows that only the formed elements of blood need be supplied to a healthy animal depleted by hemorrhage.

The essential peculiarities of the red blood cells must be taken into account in any attempt to preserve them. They have little of the ability to adjust themselves to changes in external conditions which is possessed by many somatic cells[2] in common with the unicellular organisms. As bits of protoplasm without a nucleus, multiplication is impossible to them, and their existence is necessarily limited, whether they are in the circulation or *in vitro*. If they are to be kept alive outside the body, it must be in what one might term a state of suspended

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[1] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 625.

[2] Striking instances of this ability are afforded by the *in vitro* growth of tissue cells in plasma diluted with distilled water, in plasma from other animal species, in synthetic media, etc.

animation.   Experience has taught that they are best kept in the cold. But even then whether the medium be serum or physiological salt solution, they break down within a few days,—much sooner than they are supposed to do in the circulation.   Evidently there is room here for improvement in method, or else current estimates of the length of life of the red cell are faulty.

Injury during Washing.

For our experiments we have used cells of the rabbit, dog, sheep, and man.   It seemed best in the initial tests to free the cells of plasma and suspend them in solutions of known constitution. Meltzer[3] has shown that red cells shaken in plasma to the slight extent necessary for defibrination break down much sooner on keeping than when the blood is allowed to clot undisturbed.   We have asked ourselves whether injury during washing might not be responsible for the brief survival of washed cells.   That in the case of dog, rabbit, and sheep cells, especially the first mentioned, some injury may occur is evident from the hemolysis frequently observed during washing even when the cells are handled most carefully.

Experiment 1.[4]   *Cells Washed and Kept in Ringer's Solution Break Down Sooner than Those Left Undisturbed in Citrated Blood.*—Dog blood was taken in Ringer's solution containing 1 per cent of sodium citrate in the proportion of 1 part of blood to 4 of the solution.   Some of the mixture was at once tubed and set aside. The cells of the remainder were washed twice with ordinary Ringer's solution and distributed in Ringer's plus citrate and in Ringer's solution, respectively, to the same amount as in the original blood-citrate mixture.   The tip of the pipette with which the washing was carried out was kept beneath the surface of the fluid, and in other ways the handling was careful.   There was no immediate hemolysis.   Tubing was done as in the case of the unwashed blood.   The preparations were kept in the ice box.

Two days later hemolysis was well marked in the preparations of washed blood. The cells of the original citrated blood were still intact.

Experiment 2.   Cells Washed and Kept in a Plasma-Locke's-Citrate Mixture Hemolyze Sooner than Those Left Unwashed in the Same Mixture.—Rabbit blood

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[3] Meltzer, S. J., *Rep. Johns Hopkins Hosp.*, 1900, ix, 135.

[4] The experiments are all specimen instances selected from a number giving the same results.

was taken in Locke's[s]-citrate,—1 part of blood to 4 of the solution, which latter contained 1 per cent of citrate,—and some of the mixture was tubed and set aside. The cells of the remainder were washed twice with Locke's fluid and to some of the cells the original supernatant fluid, a mixture of plasma and Locke's-citrate, was restored, in the original proportion, and tubing done as usual.

After five days in the ice box there was well marked hemolysis of the washed red cells but none of those left undisturbed.

Experiment 3. Red Cells That Break Down Rapidly when Shaken in Locke's Solution Remain Intact in Plasma-Locke's-Citrate. The Plasma is the Protective Agent.—Some blood was taken from two normal sheep, two rabbits, two dogs, and two men into Ringer-citrate, in the usual 1:4 proportion. Half of each mixture was washed twice with Ringer's solution and the original volume restored with it. There was no hemolysis. The washed and unwashed specimens of blood were now placed in shaking tubes of uniform size. The same considerable air space was left in each tube and duplicate preparations were made. All were now shaken in a machine for 15 minutes, the tubes centrifugalized, and the amount of hemolysis read.

The washed human bloods shaken in Ringer's solution showed each a trace of hemolysis, the two sheep bloods considerably more, an amount which may be indicated by +, the rabbit bloods + + +, and the dog bloods + + + and + + + +, respectively. There was no hemolysis in any of the tubes containing plasma-Ringer's-citrate. The duplicate preparations confirmed these results.

As a corollary to this experiment, tests were made to see whether Ringer's-citrate without plasma had a protective action. Washed cells shaken in it went to pieces with the same rapidity as in ordinary Ringer's solution.

It is evident that the handling of red cells in salt solution, even to the small extent necessary to wash them, may be very injurious. Much of the injury is immediate and mechanical in character. Plasma has a notable influence to prevent it.

Protection.

From these observations it is plain that if washed red cells are to be properly preserved they must be protected during washing. Plasma obviously cannot be used for this purpose. Some simple agent is needed. And this was found in gelatin. $\frac{1}{8}$ to $\frac{1}{4}$ per cent of gelatin in Locke's solution protects cells absolutely against injury during washing, and even during prolonged shaking.

[s] The Locke's solution referred to here and elsewhere is Locke's modification of Ringer's fluid, but without any sugar: 9.2 gm. sodium chloride, 0.05 gm. sodium bicarbonate, 0.1 gm. potassium chloride, 0.1 gm. calcium chloride in 1,000 cc. of water.

Experiment 4. The Protective Influence of Gelatin.—Dog blood was taken in Locke's-citrate as usual (1 part to 4), distributed in equal quantity in eight tubes, and centrifugalized at high speed. From all except one of the tubes the supernatant fluid was now pipetted off as completely as possible, by means of a capillary pipette. The original volume was restored to six tubes with Locke's solution containing graded amounts of gelatin, from $\frac{1}{8}$ per cent to $\frac{1}{16}$ per cent, while to the seventh tube ordinary Locke's solution was added. All were now stoppered, shaken in a machine for 15 minutes, centrifugalized, and the hemolysis was noted. Shaking was then renewed for 15 minutes, and the tubes were placed in the ice box and examined after 3 days. The results are given in Table I.

TABLE I.

Hemolysis.

Time of shaking.	Locke's solution	+ $\frac{1}{16}$ per cent gelatin.	+ $\frac{1}{12}$ per cent gelatin.	+ $\frac{1}{10}$ per cent gelatin.	+ $\frac{1}{8}$ per cent gelatin.	+ $\frac{1}{4}$ per cent gelatin.	+ $\frac{1}{2}$ per cent gelatin.	Citrated blood.
min.								
15........	+++	+++	+++	+−	0	0	0	0
30........	+++	+++	+++	+−	0	0	0	0

Many subsequent experiments confirm this one. The protection afforded by the addition of $\frac{1}{8}$ per cent of gelatin to Locke's solution is for practical purposes perfect, and we have employed it regularly when blood was to be washed. Cells of the sheep, dog, and rabbit thus protected last days longer than when handled in ordinary Locke's solution.

Specific Differences in Fragility.

Protection is especially needed in the case of the red cells of the dog. Ottenberg, Kaliski, and Friedman[6] in some experiments on the normal hemagglutinins of this animal found that erythrocytes washed and placed in salt solution broke down too rapidly to be used. They were obliged to employ cells suspended in their own serum. Usually we have noted an abundant hemolysis within a few hours of dog cells washed after the ordinary methods. But if gelatin-Locke's be the washing medium, they may remain intact for several days. The erythrocytes from different dogs show marked differences in their period of survival.

[6] Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *Jour. Med. Research*, 1913, xxviii, 141.

It has proved interesting to compare the resistance to mechanical injury (shaking) of the cells of different species. The results obtained in Experiment 3 express a general rule. Human cells have by far the greatest resistance. In their case, as we have repeatedly found, washing with Locke's solution after the ordinary method entails no perceptible injury, the cells remaining unhemolyzed as long as when gelatin-Locke's is used. Sheep cells come next in point of endurance. But washing in ordinary Locke's solution injures them somewhat, and it affects much more considerably rabbit cells and dog cells. All of these require protection by gelatin. An absolute scale of the fragility of bloods is difficult to prepare because marked variations are observed with the cells of different individuals and with the length of time that shaking is carried on. Dog blood at first breaks down far more than rabbit blood, but as shaking is continued the latter shows the greater destruction. In order properly to illustrate the findings, curves should be constructed like those that Smith and Brown[7] have used to record the percentages of erythrocytes breaking down in salt solutions of graduated hypotonicity.

Resistance to Shaking versus Resistance to Hypotonic Solutions.

Has the resistance of erythrocytes to mechanical injury any relation to their behavior in hypotonic salt solution?

TABLE II.

	Sheep C.	Sheep M. A.	Dog B.	Dog Dal.
Minimum resistance in salt solution=	0.76 per cent.	0.70 per cent.	0.58 per cent.	0.50 per cent.
Hemolysis on shaking.	Tr.	++	++++	++++

Experiment 5. The Resistance of Erythrocytes to Hypotonic Salt Solution, and to Shaking Vary Independently.—Blood was taken from two dogs often bled previously and from two sheep immunized against a bacterium and also bled often. 15 cc. of each blood were allowed to flow into an equal amount of a solution containing 4 per cent of sodium citrate, 0.6 per cent of sodium chloride, and the other salts of Locke's solution in the usual amount.

[7] Smith, T., and Brown, H. R., *Jour. Med. Research*, 1906, xv, 425.

Each specimen was divided into two equal parts; these were washed twice with Locke's fluid, and one was made up with it as a 10 per cent suspension for shaking, while the other, in thick suspension, was used for tests of resistance to salt solution. Shaking was carried on for 10 minutes and the tubes were centrifugalized and read. Two drops of the thick suspension were added to a number of tubes containing 3 cc. of hypotonic sodium chloride solution. The tubes of hypotonic salt solution were so prepared that each differed from the next by 0.02 per cent of sodium chloride. Readings were taken from these after 3 hours and again after 12 hours.

The results are shown in Table II. The percentages of salt solution recorded are those giving the faintest trace of hemolysis.

In this experiment the cells of the sheep were far less resistant to hypotonic salt solution than those of the dogs, but to shaking they exhibited much the greater resistance. The same phenomenon was evident in the case of individuals of one species (Sheep C. and M. A.). This inverse relationship between the two resistances does not always hold. Human erythrocytes, as we have found, are very resistant to shaking and quite resistant to hypotonic solutions. Hamburger[8] has pointed out that resistance to hypotonic salt solution is the result of many factors, and Rywosch[9] and Rous[10] respectively have proved that it is independent of resistance to chemical hemolysins and to a specific serum hemolysin. The fact that it is also independent of resistance to mechanical injury shows that the term fragility, so often used in connection with it, is a misnomer. Resistance to hypotonic solution is in no real sense an index to the fragility of red cells. A clinical investigation of this fragility as determined by shaking experiments might be not without importance.

Protection versus Preservation.

The injury sustained by cells washed and kept in salt solution is one cause for their rapid breaking down, but it is not the only one.

Experiment 6. Locke's Solution Is Injurious to Cells Kept in It after Washing.—Dog blood was taken as usual in citrate and twice washed, part with Locke's

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[8] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[9] Rywosch, D., *Arch. f. Physiol.*, 1906–07, cxvi, 229.

[10] Rous, P., *Jour. Exper. Med.*, 1909, xi, 763.

solution and part with Locke's solution containing ½ per cent of gelatin.   The fluid was taken off, as far as possible, by means of a capillary pipette, and the cells were made to 12½ per cent suspension with ordinary Locke's.   1 cc. of each suspension was now added to 3 cc. of the original plasma-Locke's-citrate and to ordinary Locke's solution, respectively.   Examination of the tubes after they had been 4 days in the ice box showed that there was no hemolysis of the cells washed in gelatin-Locke's and kept in plasma-Locke's-citrate, and only the faintest trace in the case of those washed in Locke's.   Cells kept in Locke's after washing in gelatin-Locke's showed a + hemolysis, and those washed in plain Locke's a + + hemolysis.

Here the protective action of gelatin is evident.   But it is also plain that, whether red cells are protected during washing or not, they break down sooner in Locke's solution than in a mixture of Locke's with plasma and sodium citrate.

Preservation.

The search for a preservative was now begun.   The form of the experiments was simple.   Blood was taken in Locke's solution containing 1 per cent of sodium citrate, and its cells were twice washed with Locke's containing ⅛ per cent of gelatin.   All possible fluid was then pipetted off, the cells made up in suspension with Locke's, and portions added to the various preservative fluids.   These had, for the most part, Locke's solution, without sugar, as a base.   We are aware that other solutions, Tyrode's for example, might have proved better; but Locke's fluid has the advantage of simplicity, and some preliminary observations suggested that cells last longer in it than in 0.95 per cent sodium chloride or in ordinary Ringer's fluid.   The substances tested for preservative action were all of high purity.   Small vials with sterile corks were used as containers, and only enough cells were placed in each to cover the bottom thinly.   This was done because it was found that cells allowed to sediment in a thick layer exhibit very slowly the action of the supernatant fluid.   So many vials were used in each experiment that it was not practicable to test the sterility of each preparation by means of culture, and instead reliance was placed on duplicate preparations, on repetition of the work, and on the graded character of the tests which ensured graded results except when some technical error was present.   The preparations were kept in the dark under aseptic conditions at a temperature of 1° to 3°C.   At first the

appearance of hemolysis was taken as the limit of the period of survival of the cells; but as the work progressed and more precise evidence was needed, it was obtained by transfusing the kept cells in bulk.   The transfusion experiments are dealt with in the second part of this paper.

The action of sodium citrate was tested out because of the results with plasma-Locke's-citrate.   But though a 1 per cent solution of it in Locke's fluid proved better for dog and rabbit corpuscles than plain Locke's solution, it had not sufficient preservative action for practical purposes.

The first step toward preservation of the cells seemed to be to obtain conditions that would not be deleterious to them.   In salt solutions such as Ringer's and Locke's the conditions are far from natural. Red cells placed in them undergo a change of form, becoming spherical (Hamburger).[11]   Weidenreich[12] attributes this to the absence of colloids.   So tests were begun with Locke's solution to which colloids of various sorts had been added.   Gelatin was first employed.   It has no preservative action, but, on the contrary, in the amount of $1\frac{1}{2}$ per cent, which restores the normal shape to the cell (Weidenreich), it causes a gradual hemolysis and browning of the blood pigment.   Washed agar, soluble starch, plain starch, and dextrin were also tried, but only the last proved useful, and that only for dog blood.   The fluid pressed from serum coagulated by heat, and serum water made up to isotonicity with the salts of Locke's gave poor results.   ·

A number of observers[13] have shown that the red cells are almost totally impermeable to sugars in contrast with other crystalloid substances.   Solutions of sugar, then, not inconceivably might act like colloids in their effect on the shape of the cells.   Sugars dissolved in Locke's fluid were accordingly tried and in them red cells were found to be preserved intact for a remarkable length of time.   Dextrose and saccharose had the most marked action, but levulose, maltose, and lactose were not far behind.   The cells still remained spherical, however.

[11] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[12] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

[13] Hedin, Hamburger, Kozawa.

The best results were obtained with mixtures of Locke's solution and isotonic solutions of the sugars in water.[14] Isotonic mixtures are better than hypertonic, though slight variations in tonicity are well borne. The small amount of sugar present in Locke's solution, when it is made up after the usual formula, is far below that which exerts a preservative influence. In isotonic sugar solutions alone erythrocytes keep fairly well, but they sediment in a firm layer which can hardly be suspended without hemolysis. The sugar solution and Locke's solution to be mixed together must be autoclaved separately, because in mixtures of them the sugar caramelizes during the autoclaving.

Optimum Preservative Fluids.

For the cells of each species a special preservative mixture is required. In our experience the optimum preservative for the washed cells of the sheep is one made by adding to Locke's solution sufficient saccharose in isotonic, watery solution to give an ultimate concentration of 2.8 per cent of saccharose. Cells twice washed in gelatin-Locke's remain unhemolyzed in this sugar medium for at least 3 or 4 weeks; and even after 2 months hemolysis is exceedingly slight. As is well known, the cells begin to break down within 3 or 4 days when washed and kept in Locke's solution in the ordinary way. For their proper preservation, it is essential that they be washed in an abundance of fluid, since a trace of plasma soon leads to hemolysis. The properly washed and kept cells retain their color; they take up and give off oxygen readily; and though they sediment into a rather firm layer they are easily made into a uniform suspension which passes readily through a filter paper. The cells in such a suspension are discrete, not stuck together. They will withstand repeated washings, but after washing they break down somewhat sooner in Locke's solution than do fresh cells. Cells kept for 3 weeks and for a month have been used for the Wassermann reaction and compared with cells freshly obtained from the same sheep. They had the same hemolytic titer and gave identical results in the reaction.[15]

[14] Saccharose 10.3 per cent, dextrose 5.4 per cent.
[15] For these tests we are indebted to Dr. Russell L. Cecil.

Dextrose is a fairly good preservative for washed sheep cells and human cells. But the latter are best kept in a saccharose-Locke's mixture containing about 5 per cent of the sugar (4.9 per cent). We have thus preserved them for 4 weeks without any hemolysis in the supernatant fluid or on washing. They are easily suspended, and like sheep cells take up and give off oxygen readily. Cells of both sorts begin to break down in about 10 days in a plasma-Locke's-citrate mixture.

Washed rabbit cells remain unhemolyzed longest in plasma-Locke's-citrate, slightly over 2 weeks as a rule. In a mixture of Locke's with glucose and saccharose, containing 3 and 6 per cent of the sugars, respectively, they stay intact from 9 to 12 days.

Dog cells are most difficult to keep. They are very frail, and hemolyze rapidly in their own plasma. In a saccharose-dextrin-Locke's fluid containing 1.6 to 2.7 per cent of the sugar and 2 per cent of dextrin, they show no breaking down for from 5 to 12 days, when they have been thoroughly washed with gelatin-Locke's before being placed in this medium. Preservation for so long a time must be regarded as notable for elements which under ordinary circumstances, washed and kept in Locke's or sodium chloride solution, begin to hemolyze at once. 2 per cent of dextrin added to the Locke's-sugar mixture aids the preservation of dog cells; but it cannot be regarded as uninjurious, since in amounts of 10 per cent it causes a gradual browning of the blood pigment to methemoglobin. This alteration is not evident with 2 per cent dextrin.

We are inclined to attribute the hemolysis that eventually occurs in the optimum preservatives to autolysis within the substance of the cells. That it is not due to the action of the preservatives themselves is well shown by comparing the results with cells allowed to settle in these fluids and cells agitated each day. In such an experiment gelatin must be present to prevent mechanical injury. If the fluid used as a preservative is harmful the cells brought in daily contact with it by agitation break down much sooner than those left to sediment. This is what happens in Locke's solution, for example. But with fluids such as the saccharose-Locke's that is optimum for washed sheep cells, the sedimented and agitated cells show the same gradual breaking down.

Concomitant Factors.

There are many factors besides the character of the preservative which conceivably may affect the period of survival of the cells. Sedimented cells lose their oxygen within a few days; they are exposed to a possible digestive action of the leukocytic pellicle, and to possible injury of the living erythrocytes by contact with dead cells scattered in the mass. Cells that are stirred remain bright and are not exposed to the dangers mentioned. But in experiments specifically directed to these points, we have found that red cells deprived of oxygen last no longer than those well supplied with it, that the leukocytic pellicle of normal blood does not cause hemolysis of the erythrocytes, and· that contact injury of living cells by the dead can be disregarded. A priori one might suppose that cells would keep best in a very small. quantity of preservative fluid, since in this there would be less loss of their diffusible substances. But tests in which the cells were stirred daily and allowed to settle through long and short columns of fluid, have failed to show that this has any importance. It is our practice to place the washed cells in five or six times their bulk of preservative fluid and allow them to remain in sediment until wanted.

Preservatives Are Not Protectives.

If the preservative solutions protect the cells against mechanical injury, gelatin can be dispensed with and a single solution used throughout.

Experiment 7. Dextrose and Dextrin Are Not Protectives.—Dog blood was caught in Locke's-citrate as usual and the mixture distributed in equal amount in 5 centrifuge tubes. The cells of 4 were washed twice with ordinary Locke's solution, Locke's containing 5 per cent of dextrin (Merck), 3 per cent of dextrose, and 1½ per cent of gelatin, respectively, and were suspended to the original blood-citrate bulk in these fluids. With the 5th tube the form of washing was twice gone through, using the original fluid. All were now corked, shaken for 15 minutes, and centrifugalized. There was no hemolysis in the tubes containing gelatin-Locke's and plasma-Locke's-citrate, but in the others it was abundant and of about the same amount in all.

Plasma possesses both protective and preservative qualities, but it is, of course, no simple fluid.

The Preservation of Leukocytes.

Some tests were made to determine whether leukocytes require the same protective and preservative media as the red cells.   An aleuronat exudate of the dog containing many large mononuclear cells was washed, half in gelatin-Locke's, half in ordinary Locke's solution, and portions were distributed in various fluids for preservation.   As a control, the red cells of the same dog were similarly treated.   After 1 week in the cold, all were washed, this time in ordinary salt solution, and the ability of the mononuclear cells to take up rat erythrocytes was tested, with fresh dog serum as complement.   Only those cells which had been placed for keeping in the original citrated plasma now showed phagocytosis, and this was independent of whether gelatin had been present in the original wash fluid.   Mononuclears kept in Locke's solution, and in the sugar-Locke's, and the·sugar-Locke's-dextrin mixtures most favorable to the red cells, failed entirely to ingest the rat corpuscles.

The Preservation of Unwashed Red Cells.

The results with red cells gave some ground for the hope that the erythrocytes of blood received directly into a medium preservative for the washed cells and thus kept, would remain unhemolyzed longer than under the usual conditions.   Experiments along this line were undertaken.   It was necessary, of course, to use some anticoagulant, and for this purpose sodium citrate was employed.   Considerable quantities of blood were taken, distributed with the preservative mixtures in large test-tubes, and kept in the cold.   To estimate hemolysis not only was the color of the supernatant fluid noted but the cells were stirred up in $\frac{1}{3}$ per cent gelatin-Locke's and centrifugalized.   This frequently brought to light a marked hemolysis of which there had been no trace when the cells lay in sediment.   Our freezing point determinations showed that a watery solution of sodium citrate containing 3.8 per cent of the salt is isotonic with 0.95 per cent sodium chloride.   Tests were first carried out for an optimum blood-citrate mixture.   .

Experiment 8.   The Optimum Blood-Citrate Mixture for Rabbit Cells.—The blood of two normal rabbits, X and Y, was taken in portions of 3 cc. into sodium

TABLE III.

Hemolysis.

Mixture. 1½ cc. blood +	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
2/6 cc. 10% sodium citrate.........	++	+++++	Clotted.	++	++		Clotted.	++++
3/6 " 10 " "	+++	+++++	++	+++	+++		++	+++
3/6 " 7.6 " "	+++	+++	+	++	+++		+	+++
3/6 " 7.6 " "	++	++	Tr.	Tr.	++		Tr.	+
3/6 " 3.8 " "	+	+	+	"	+		+	Tr.
1 " 3.8 " "	Tr.	+−	Ft. Tr.	F'test. Tr.	+		0	"
1½ " 3.8 " "			0				0	

citrate solutions of various concentration and amount. Each mixture was divided into two equal portions and tubed. After many days one tube of each sort was tested for hemolysis, and still later (after 47 days, all told, in the case of Y) the second was examined. The color of the supernatant fluid was recorded and also the amount of hemolysis when the cells were suspended in 6 cc. of ⅛ per cent gelatin-Locke's and centrifugalized.

The results are shown in Table III.

As this experiment shows, the preservation of the erythrocytes of citrated rabbit blood is much influenced by the amount and concentration of the citrate. We have repeatedly found that the best results are obtained, not with the smallest amount of citrate that will prevent clotting, but with equal parts of blood and isotonic citrate solution. The same amount of a somewhat hypotonic solution (2.5 per cent citrate) also gives good results. With human blood, on the other hand, the proportion and concentration of the citrate seem to have little influence. With sheep and dog blood no quantitative tests have been made.

As Experiment 8 shows, when rabbit blood is mixed with the right amount of citrate its cells remain intact for a long time. The addition of sugars further increases the preservation and the real length of life of the cells to a slight extent, as our transfusion experiments have shown. Sheep cells keep no longer in the presence of sugar. But for human blood cells, it has a remarkable preservative effect

Experiment 9. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—20 cc. of two human bloods were taken, that from X in an equal bulk of 2½ per cent citrate in water, that from Y in a citrate-salt solution containing 2 per cent of citrate, 0.3 per cent of sodium chloride, and the other salts of Locke's in the same relative proportion. To equal amounts of the blood-citrate mixtures, Locke's solution and isotonic saccharose and dextrose solutions, respectively, were added. One tube of each of these preparations was examined after 13 days and another after 20 days. The sedimented corpuscles were tested for concealed hemolysis by suspending them in ⅛ per cent gelatin-Locke's and immediately centrifugalizing. The results are given in Table IV.

Experiment 10. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—Three human bloods were taken in various amounts of citrate, as in the case of Experiment 8 with rabbit bloods. To one of the mixtures which contained 3 parts of blood and 2 parts of isotonic (3.8 per cent) citrate solution, 5 parts of isotonic dextrose solution were added. After 4 weeks all the preparations were examined as usual, the cells being suspended in gelatin-Locke's and centrifugal-

TABLE IV.

Hemolysis.

Mixture.	Blood X. 13 days.		Blood Y. 13 days.		Blood X. 20 days.		Blood Y. 20 days.	
3 parts citrated blood +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	+−	+−	Tr.	+−			Tr.	+
1 part Locke's.	Tr.	+−	+−	+−	Tr.	++−	+	+
3 " "	+−	+−	++−	+−	++−	++	++−	++
7 " "	++−	++	+++	+++	++	++++	++	+++
1 " saccharose.	0	Tr.	0	+−	Ft. Tr.	+−	Ftest. Tr.	+
3 " "	0	Ft. Tr.	0	+−	0	Tr.	0	+−
5 " " } 2 " Locke's.}	Ft. Tr.	" "	0	+−	0	Tr.	0	+−
1 " dextrose.	0	" "	0	Ftest. Tr.	Ft. Tr.	0	Tr.	Tr.
3 " "	0	0	0	0	" "	0	Ft. Tr.	0
5 " " } 2 " Locke's.}	0	0	0	0	" "	0	+	+−

ized. There was no hemolysis of those kept with dextrose, but those of the citrate mixtures all showed a marked breaking down, independent apparently of the amount of citrate present.

Other experiments confirm these results. It is safe to say that the red cells of normal human beings can be kept intact for nearly or quite 4 weeks, when 3 parts of the blood are taken directly in a mixture of 2 parts of isotonic sodium citrate and 5 parts of isotonic dextrose solution. With citrate alone, in any quantity, hemolysis is well marked in less than 2 weeks.

Locke's Solution Is Injurious.

The action of Locke's solution to cause hemolysis, which is so plain in the case of washed cells kept in it, is no less evident when this solution is added to citrated blood. Experiment 9 illustrates this fact for human blood.

Experiment 11. The Injurious Effect of Locke's Solution on the Cells of Citrated Rabbit Blood.—The blood of two rabbits, X and Y, was taken in an isotonic citrate solution, and to portions of the mixtures, Locke's solution and an isotonic watery solution of saccharose, respectively, were added. The tubes were examined for hemolysis after many days had elapsed (Table V).

TABLE V.

Hemolysis.

Mixture.	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
3 cc. blood + 2 cc. citrate +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	+	+	Ft. Tr.	Tr.	+		0	Tr.
3½ cc. Locke's.	++++	+−	+++	"	++++		+++	+
3½ cc. saccharose.	+	Tr.	0	0	+		Ft. Tr.	0

The greater the amount of Locke's solution mixed with the citrated blood the greater is the destruction of the corpuscles (Experiment 9). But as in the case of washed cells, the injurious action of the Locke's fluid can be completely prevented by means of a sugar.

Experiment 12. The Effect of Saccharose To Prevent the Injurious Action of Locke's Solution on Rabbit Corpuscles.—Rabbit blood was taken into isotonic citrate and to portions of the mixture Locke's solution, isotonic saccharose solution, and combinations of the two were added. After many days the tubes were examined for hemolysis in the usual way (Table VI).

TABLE VI.

Hemolysis.

Mixture.	Percentage in ultimate mixture.				Rabbit X. 26 days.		Rabbit Y. 34 days.	
3 cc. blood + 2 cc. citrate +	Blood.	Citrate.	Sugar.	Locke's.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	60	40	–	–	+	+	Ft. Tr.	Tr.
3½ cc. saccharose.	36	24	40	–	+	Tr.	0	0
3½ cc. Locke's.	36	24	–	40	++++	+−	+++	Tr.
8½ cc. Locke's. 9½ cc. saccharose.	13	9	41	37	+	Ft. Tr.	0	0
17 cc. Locke's. 19 cc. saccharose.	7	5	46	42	Tr.	0	0	Tr.

A similar action of dextrose is indicated in Experiment 9 with citrated human blood.

SUMMARY.

The erythrocytes of some species are much damaged when handled in salt solutions, as in washing with the centrifuge after the ordinary method. The injury is mechanical in character. It may express itself in hemolysis only after the cells have been kept for some days. It is greatest in the case of dog corpuscles, and well marked with sheep and rabbit cells. The fragility of the red cells, as indicated by washing or shaking them in salt solution is different, not only for different species, but for different individuals. It varies independently of the resistance to hypotonic solutions.

The protection of fragile erythrocytes during washing is essential if they are to be preserved *in vitro* for any considerable time. The addition of a little gelatin ($\frac{1}{8}$ per cent) to the wash fluid suffices for this purpose, and by its use the period of survival in salt solutions of washed rabbit, sheep, and dog cells is greatly prolonged. Plasma, like gelatin, has marked protective properties.

Though gelatin acts as a protective for red cells it is not preservative of them in the real sense. Cells do not last longer when it is added to the fluids in which they are kept. Locke's solution, though better probably than Ringer's solution, or a sodium chloride solution, as a medium in which to keep red cells, is ultimately harmful. The addition of innocuous colloids does not improve it. But the sugars, especially dextrose and saccharose, have a remarkable power to prevent its injurious action, and they possess, in addition, preservative qualities. Cells washed in gelatin-Locke's and placed in a mixture of Locke's solution with an isotonic, watery solution of a sugar remain intact for a long time,—nearly 2 months in the case of sheep cells. The kept cells go easily into suspension free of clumps, they pass readily through paper filters, take up and give off oxygen, and when used for the Wassermann reaction behave exactly as do fresh cells of the same individual. The best preservative solutions are approximately isotonic with the blood serum. If the cells are to be much handled gelatin should be present, for the sugars do not protect against mechanical injury.

Different preservative mixtures are required for the cells of different species. Dog cells last longest in fluids containing dextrin as well as a sugar. The mixture best for red cells is not necessarily best for leukocytes.

A simple and practical method of keeping rabbit and human erythrocytes is in citrated whole blood to which sugar solution is added. In citrated blood, as such, human red cells tend to break down rather rapidly, no matter what the proportion of citrate. Hemolysis is well marked after little more than a week. But in a mixture of 3 parts of human blood, 2 parts of isotonic citrate solution (3.8 per cent sodium citrate in water), and 5 parts of isotonic dextrose solution (5.4 per cent dextrose in water), the cells remain intact for about 4 weeks. Rabbit red cells can be kept for more than 3 weeks in citrated blood; and the addition of sugar lengthens the preservation only a little. The results differ strikingly with the amount of citrate employed. Hemolysis occurs relatively early when the smallest quantity is used that will prevent clotting. The optimum mixture has 3 parts of rabbit blood to 2 of isotonic citrate solution.

In the second part of this paper experiments are detailed which prove that cells preserved by the methods here recorded function excellently when reintroduced into the body.

DISCUSSION.

Our findings show that in experiments with cells washed in salt solutions there is a large source of possible error in injury done during washing. That it has been so long overlooked by investigators is probably due to the fact that mechanical injury does not alter hemolytic titer, and furthermore that such injury often manifests itself only after the cells have been kept for several days. The reasons for the injury in salt solution and for the protective action of gelatin are not clear. We were led to employ the latter through some observations of Weidenreich,[16] who showed that red cells placed in salt solution containing gelatin do not become spherical as in ordinary salt solution but retain their normal shape. From this he concluded that the shape of the cell is determined not only by the osmotic pressure of the surrounding fluid, but by its molecular force (*Molecularkraft*), as determined through its content in colloidal substances. But the amount of gelatin which will confer protection in salt solution is far too little appreciably to alter the molecular force, or, as we have found, to preserve the normal shape of the cell. For this latter effect, at least

[16] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

as much as the 1½ per cent of gelatin, recommended by Weidenreich, is necessary. In Locke's solution containing ⅛ per cent of gelatin the cells are spherical. In the preservative sugar-Locke's solutions also the cells are spherical. It is interesting that cells kept for many days in such distorted condition should retain their usefulness for the body.

The ability of plasma to protect red cells against mechanical injury may explain to some extent the relatively long survival of the latter in the circulation. A clinical investigation of the mechanical fragility of red cells as determined by shaking experiments might yield results of value.

The preservative action of the sugars on red cells kept *in vitro* is largely dependent, when Locke's solution is present, on a prevention of injury from this latter. That Locke's solution should fail to be a physiological medium for red cells is scarcely surprising. Much recent work has gone to show that for the cells of different organs, different solutions are physiological. But Locke's solution is not merely lacking in some constituent needed by the red cells. It is actively injurious. This is well seen in the experiments with citrated blood, as also in the action of sugars to prevent the injury. The further preservative action of the sugars is perhaps referable to their ability to retard proteolytic digestion;[17] and the peculiarity that it is effective only in the case of certain red cells, to the fact that the erythrocytes of different species have a somewhat different permeability for sugars.[18]

Sheep cells washed and kept according to our methods are as suitable for the Wassermann reaction as fresh cells. But considerable manipulation of them is required. Formalization of the defibrinated blood, as practiced by Armand-Delille and Launoy,[19] and by Bernstein and Kaliski,[20] would seem more practical. The blood-citrate-saccharose mixture should prove useful for the preservation of rabbit and human corpuscles for culture media.

[17] Ogáta, M., *Arch. f. Hyg.*, 1885, iii, 204.

[18] Kozawa, S., *Biochem. Ztschr.*, 1914, lx, 146, 231.

[19] Armand-Delille, P., and Launoy, L., *Ann. de l'Inst. Pasteur*, 1911, xxv, 222.

[20] Bernstein, E. P., and Kaliski, D. J., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xli, 490.

THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

II. The Transfusion of Kept Cells.

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In the foregoing paper methods are described whereby red blood cells may be kept intact for long periods *in vitro*. It has remained to determine whether cells kept according to these methods are alive in the sense that they are capable of functioning in the animal body. This can be told by transfusion of the kept cells in bulk, with appropriate control. We have performed many such experiments, using rabbits.

The cells for preservation were obtained by bleeding etherized rabbits from the carotid or aorta into a Locke's-citrate mixture or an isotonic solution of citrate (3.8 per cent) in water. In some instances, the cells were washed, with gelatin-Locke's ($\frac{1}{8}$ per cent gelatin), and placed in a preservative solution; in others they were kept, without washing, in the original mixture of citrate and blood, or in citrate and blood plus a sugar. When they were to be used the supernatant fluid was pipetted away; and sometimes the sediment was suspended directly in Locke's solution to the original blood bulk and used for injection. More often it was washed once or twice with gelatin-Locke's and then suspended in ordinary Locke's. The suspension was filtered through two thicknesses of gauze; warmed to body temperature; and introduced under slight pressure into the ear vein of a rabbit which had just been bled.

The rabbits which furnished and received the cells were selected from a number tested against one another to rule out the presence of iso-agglutinins and isolysins. In the rabbit such antibodies are weak and infrequent. None of our results suggest their action. The animals chosen as recipients were bled in large amount from the ear

127

and the hemoglobin loss was replaced by the transfusion of kept cells. The bleeding and injection were repeated if the amount of kept cells allowed, as in instances when the cells from two rabbits had been preserved for the injection of a single individual. In every case a large proportion of the blood was drawn and replaced. The fate of the transfused cells was followed by blood counts, hemoglobin estimations, direct microscopic observations on the appearance of the blood elements, and by daily tests of the urine for hemoglobin (guaiac and spectroscopic tests) and bile (Gmelin and Bouvais tests). In addition, the percentage of reticulated red cells in circulation was noted in wet preparations stained with cresyl blue. This was done because an increase in the reticulated cells indicates an abnormal activity on the part of the bone marrow, such as would be the result of an increased destruction of the circulating erythrocytes. The rabbit's temperature was taken twice daily and its weight frequently recorded.

Some of the results may be briefly summarized and thus a repetition of data avoided. The bleeding and replacement of blood were always well borne. They took from one-half to three-quarters of an hour, and at the conclusion the animal usually went at once to eating. There was never a suppression of urine. Bile was never found in it and blood only in one instance, in a control experiment. The rabbit was, in this case, injected with cells kept, not in a preservative, but in Locke's solution, and a copious breaking down resulted with hemoglobinemia, hemoglobinuria, and death (Experiment 6).

In the specimen protocols that follow, the amount of blood drawn and replaced is recorded in percentage of the animal's total hemoglobin content. This is not to be confused with the percentage of hemoglobin in the circulating blood as obtained with the hemoglobinometer. It gives a much more precise idea of the proportion of formed elements dealt with, than would the blood volume. As is well known, when bleeding is done, even very rapidly, the last portion of blood contains much fewer cells than the first.[1] In thirteen instances in which we have taken into citrate from 30 to 50 per cent of the total calculated volume of a rabbit's blood and have com-

[1] Boycott, A. E., in Pembrey, M. S., and Ritchie, J., Text-Book of General Pathology, New York and London, 1913, 9.

pared the hemoglobin concentration of the drawn blood with that of the animal originally, it was found to be on the average 17 points lower by the Sahli scale. In general we have noted both the volume and the hemoglobin of the drawn blood, and have calculated from these the amount of cell suspension of known hemoglobin content that should be injected to restore the animal's hemoglobin content to the normal. In some of the early experiments this was not done and replacement was made by volume. According to Boycott and Douglas[2] a rabbit possesses 5.5 cc. of blood for 100 gm. of body weight. We have used this figure with hemoglobin estimations by the Sahli scale in determining the proportion of total blood pigment, and thus of erythrocytes, drawn and replaced. Since only 93 per cent of a rabbit's total hemoglobin is in the circulating blood (Boycott

TABLE I.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.
		per cent
Before.....................	4,890,000	87
3 hrs. after...............	4,590,000	85
24 " "	4,460,000	85
3 days "	4,510,000	83
6 " "	4,450,000	81

and Douglas), our estimate must be considered as rather less than the actual proportion. When two withdrawals and injections were carried out, allowance was made in the calculations for the mixed character of the second portion of blood withdrawn.

Experiment 1. Transfusion with Washed Cells Kept in a Dextrose-Locke's Solution and Washed before Use.—An animal weighing 1,700 gm. was bled 41 cc., and 41 cc. of a suspension of kept blood cells at once injected. By calculation some 30 per cent of the total hemoglobin was thus taken and replaced. The kept blood had been taken eight days previously, twice washed, and placed in Locke's solution to which 2½ per cent of glucose and ⅛ per cent of gelatin had been added. It was washed twice just previous to injection and suspended in Locke's solution. This handling brought about no hemolysis (Table I).

² Boycott, A. E., and Douglas, C. G., *Jour. Path. and Bacteriol.*, 1909, xiii, 256.

The animal was killed on the sixth day because of an infected ear. The reticulated red cells were not followed in this case. Fresh preparations of the blood failed to show shadows.

Experiment 2. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Washed before Use.—For this experiment the erythrocytes of two rabbits were used after they had been kept separately in mixtures of the citrated blood with saccharose for 11 and 12 days, respectively. Three parts of the blood were mixed for keeping with 2 of isotonic citrate and 5 of isotonic saccharose solution. The cells were washed once just before injection and suspended in Locke's solution. Following the injection there was no hemoglobin in the supernatant or wash fluid. The rabbit that acted as recipient was twice bled (40 and 6 cc.) and twice injected. 31 per cent of the animal's hemoglobin was thus replaced (Table II).

TABLE II.

Time before or after transfusion.	Red blood corpuscles.	Hemo-globin.	Reticulated cells.	Weight.	Urine.	Temper-ature.
		per cent		*gm.*	*cc.*	*°F.*
1 day before..................	6,500,000	91	25 in 500	2,025	65	102
Just before...................	6,100,000	90	19 " "		38?	102
3 hrs. after..................	6,500,000	90				
1 day "	6,640,000	87	24 " "		150	102.6
2 days "	6,540,000	86	14 " "		80	101.9
3 " "	6,540,000	83	12 " "	2,050	150	101.9
4 " "	6,840,000	91	13 " "		180	101.6
5 " "	6,420,000	85	11 " "		353	101.9
7 " "	6,730,000	89	9 " "		110	101.8
9 " "	6,640,000	90	11 " ".	1,875	80	101.9

Fresh preparations of the blood made daily appeared normal throughout. On the third day after the transfusion a slight polychromatophilia was noted.

Experiment 3. Transfusion with Cells Kept in a Blood-Citrate Mixture and Washed before Use.—The blood of two rabbits was taken into citrate as usual (3 parts of blood to 2 parts of citrate) and kept for 11 days. The cells were washed once just prior to injection and suspended in Locke's solution. There was a slight hemolysis in the supernatant and wash fluids: The amount was calculated by comparing the color of these fluids with a laked preparation of the ultimate suspension, and it was found to equal about 0.2 per cent of this suspension. The recipient rabbit was bled 60 cc., or 36 per cent of the total hemoglobin content, and an equivalent amount was injected (Table III).

Throughout in fresh preparations the blood appeared normal. On the second day after transfusion occasional polychromatophilia was noted.

TABLE III.

Time before or after transfusion.	Red blood corpuscles.	Hemo-globin.	Reticulated cells.	Weight.	Urine.	Temper-ature.
		per cent		gm.	cc.	°F.
3 days before...............	6,320,000	87	12 in 500	2,250		101.8
1 day "	6,480,000	85	14 " "		70	101 8
Just "	6,010,000	80	18 " "		35	102.0
5 hrs. after...............	6,460,000	85				
1 day "	6,380,000	86	9 " "		60	101.4
2 days "	6,820,000	90	16 " "		35	102.2
3 " "	6,820,000	86	11 " "		64	102.2
4 " "	6,440,000	86	6 " "		51	101.9
5 " "	6,560,000	87	8 " "	2,375	140	101.5
7 " "	6,850,000	86	10 " "		250	101.2
9 " "	7,390,000	93	5 " "		172	101.4
11 " "	6,720,000	88	6 " "	2,300	170	102.0

Experiment 4. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Suspended Directly in Locke's Solution.—The blood of two rabbits was taken and kept as in Experiment 14 for 13 and 15 days, respectively. The supernatant fluid was pipetted off just prior to injection and the cells suspended in Locke's solution without washing. Two bleedings (of 40 and 50 cc.) were done and two injections. About 64 per cent of the total hemoglobin was thus withdrawn and more than replaced, as the blood examination showed (Table IV).

On the 2nd day after operation there were slight anisocytosis and polychroma-tophilia.

TABLE IV.

Time before or after transfusion.	Red blood corpuscles.	Hemo-globin.	Reticulated cells.	Weight.	Urine.	Temper-ature.
		per cent		gm	cc.	°F.
1 day before...............	4,860,000	68	16 in 500	1,800		103.4
Just "	4,790,000	65	20 " "		150	103.2
3 hrs. after...............	5,670,000	81				
1 day "	5,220,000	76	16 " "		130	102.6
2 days "	• 5,280,000	73	21 " "		10?	103.0
4 " "	5,360,000	75	19 " "	1,825	170	102.4

Many experiments similar to these were done and with the same general results. Rabbit red blood cells kept for two weeks *in vitro* under suitable conditions can be used with good results to replace the blood lost in a hemorrhage. It is unnecessary to wash the cells which

may be simply suspended in Locke's solution after the preservative mixture is pipetted off.   The preservative mixture which we have found best,—blood plus sodium citrate plus an isotonic saccharose solution—cannot be injected with the cells because of its content in citrate, but the small portion of it that remains with the cells after pipetting is not harmful.   In blood-citrate mixtures without sugar the cells show some slight hemolysis (Experiment 2) after 2 weeks; and in several instances a slight drop in the cell count and hemoglobin percentage following transfusion, together with a rise in the number of reticulated red cells, has indicated that the kept cells were disappearing from the circulation and that the bone marrow was active in repairing the loss.

TABLE V.

	Time.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.
			per cent	
Rabbit A.				
35 cc. taken of the total 78 cc. of blood............	Before operation.	5,500,000	106	2 in 500
	Day after "	2,400,000	48	53 " "
	19 days after "	5,000,000	77	27 " "
Rabbit B.				
45 cc. taken of the total 139 cc. of blood............	Before operation.	6,100,000	112	1 " "
	Day after "	3,600,000	65	15 " "
	18 days after "	5,870,000	110	14 " "
Rabbit C.				
42 cc. taken of the total 94 cc. of blood............	Before operation.	6,800,000	125	2 " "
	Day after "	3,000,000	54	13 " "
	25 days after "	6,000,000	112	35 " "

In striking contrast to these results are some that were obtained in control experiments.

Experiment 5.   Effects of Bleeding Alone.—Two rabbits were bled as usual, but received no injection afterwards.   Both died within a few minutes.   The calculated blood volume of the animals was 88 and 60 cc., and the bleedings were for 50 and 28 cc., respectively.

Experiment 6.   Effects of Bleeding Followed by Injection of Locke's Solution.— Three rabbits were bled and an equivalent amount of Locke's solution was injected intravenously.   There was an immediate great drop in hemoglobin percentage and number of red cells.   Regeneration was still incomplete after many days (Table V).

Experiment 7. Bleeding Followed by Transfusion of Cells Washed and Kept in Locke's Solution.—A rabbit with a calculated blood volume of 87 cc. was twice bled (44 and 28 cc.) and an equivalent amount of kept cells was introduced. Thus about 56 per cent of the total hemoglobin was replaced. The kept cells had been washed and preserved in Locke's solution for 11 days and they were again washed just previous to injection. At this time some hemolysis was noted. The animal died in less than 24 hours after the injection and on autopsy there were hemoglobinuria, hemoglobinemia, spodogenous spleen, and other findings typical of the breaking down of blood in large quantities.

These control rabbits all fared badly. Evidently the aid rendered by a transfusion of cells kept in a proper preservative is a real one. As Experiments 4 and 5 show, these cells function normally even after they have been kept *in vitro* for 2 weeks. We have performed a number of transfusions with cells kept longer. They remain unhemolyzed for as long as 4 weeks, but by the end of the 3rd week have largely lost their ability to be useful when reintroduced into the body, as shown by the fact that within a few days they disappear from the circulation. This disappearance is unaccompanied by any signs of hemolysis or, indeed, of other derangement. The animal eats well and may gain weight. The anatomical findings in such cases have interest as bearing on methods of blood destruction. Discussion of them will be reserved for another paper. From among the many experiments one will be given here to illustrate the facility with which the body disposes of blood elements no longer useful in the circulation.

Experiment 8. Transfusion with Cells Preserved Too Long in Vitro.—A rabbit weighing 1,925 gm. was bled 54 cc. and transfused with kept cells. 44 per cent (actual) of the total hemoglobin was thus taken and an amount equal to only about $37\frac{1}{2}$ per cent put back. By calculation this should have caused a fall in hemoglobin percentage as determined with the Sahli instrument to 75 per cent after the transfusion, and that indeed was the figure obtained. The cells had been kept for 23 days in a mixture of 3 parts of blood, 2 parts of isotonic citrate solution, and 5 parts of isotonic saccharose solution. For injection they were washed once in gelatin-Locke's solution and suspended in ordinary Locke's. Neither the supernatant nor the wash fluids showed the slightest trace of hemolysis (Table VI).

There was at no time bile or blood in the urine. On the 2nd and 3rd days after the transfusion, there was marked polychromatophilia and some anisocytosis. Otherwise the animal seemed normal. The blood examinations on the 2nd day indicated that nearly all the transfused cells had disappeared from the

TABLE VI.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	*°F.*
4 days before................	5,960,000	78	9 in 500			101.6
3 " "	5,850,000	81	12 " "	1,925	20?	101.8
1 day "					280	
Just "	5,810,000	80	8 " "			102.7
3 hrs. after................	5,490,000	75				
1 day "	4,380,000	58	52 " "		100	102.2
2 days "	3,890,000	51	45 " "		120	101.8
3 " "	3,900,000	59	44 " "	1,790	20	101.4

circulation. The rabbit was killed on the 3rd day. The organs appeared normal. The spleen weighed only 0.7 gm. It gave a well marked iron reaction, and showed many phagocyted red cells.

A transfusion such as the above with intact cells kept too long *in vitro* is not helpful, or only indirectly so as supplying the constituents for new red cells; but in our experience it is not harmful.

The Preservation of Human Red Cells.

Human red cells can be preserved *in vitro* much longer than rabbit cells; and there seems little doubt that they could be profitably used for transfusion. Recently Weil[3] has reported upon a number of transfusions with whole citrated human bloods kept in the cold for several days. He employed 1 part of 10 per cent citrate to 10 parts of blood. But Lewisohn[4] has shown that citrate in this amount is dangerous to the organism; and we have found that human blood thus kept with citrate begins to hemolyze in about a week, the hemolysis being more dangerous because it is often completely concealed amid the sedimented corpuscles. Cells kept in a blood-citrate-dextrose mixture, according to the method detailed in the first part of this work,[5] remain

[3] Weil, R., *Jour. Am. Med. Assn.*, 1915, lxiv, 425.

[4] Lewisohn, R., *Surg., Gynec. and Obst.*, 1915, xxi, 37.

[5] Three parts of blood are caught in a mixture of 2 parts of isotonic sodium citrate solution (3.8 per cent) in water, and 5 parts of isotonic dextrose solution (5.4 per cent) in water. The preparation is allowed to remain undisturbed in the ice box until wanted, when the supernatant fluid is drawn off and the sedi-

intact for 4 weeks; and it would seem preferable to use cells thus kept, suspending them for injection in a little salt solution. By this method the plasma is lost; and it is of great importance for some conditions in which transfusion is employed. But in cases of simple exsanguination, as Abel and his coworkers have shown,[6] to furnish corpuscles to the body is sufficient. One might ask whether the slight remnant of plasma, sugar, and citrate remaining with the corpuscles and injected with them would be harmful. Our experiments with rabbits, and recent work on the injection of citrate and concentrated sugar solutions[7] prove this danger to be negligible. The experiments with rabbits show that if blood cells are kept too long to function, but are still intact when restored to the circulation, they are easily disposed of by the body. Tests for iso-agglutinins and hemolysins would be necessary before the transfusion of kept human cells.

SUMMARY.

In order to determine the availability for functional uses of red cells kept *in vitro* by our methods, transfusion experiments have been carried out with rabbits by which a large part of their blood was replaced with kept rabbit cells suspended in Locke's solution. It has been found that erythrocytes preserved in mixtures of blood, sodium citrate, saccharose, and water for 14 days, and used to replace normal blood, will remain in circulation and function so well that the animal shows no disturbance, and the blood count, hemoglobin, and percentage of reticulated red cells remain unvaried. Cells kept for longer periods, though intact and apparently unchanged when transfused, soon leave the circulation. Animals in which this disappear-

ment of cells suspended in Locke's solution. The slight whitish flocculus which is sometimes present above the sediment disappears in the Locke's solution. It has been our practice to filter the suspensions of kept rabbit cells through gauze, previous to use. Needless to say, the preparation must be kept sterile. The sugar and citrate solutions should be autoclaved separately, or the mixture of them can be put through a Berkefeld filter.

[6] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 625.

[7] Hustin, A., *Ann. et bull. Soc. roy. d. sc. méd. de Bruxelles*, 1914, lxxii, 104. Enriquez, *Presse méd.*, 1914, xxii, 121.

ance of cells is taking place on a large scale, remain healthy save for the progressing anemia.   The experiments prove that, in the exsangui-nated rabbit at least, transfusions of cells kept for a long time *in vitro* may be used to replace the blood lost, and that when the cells have been kept too long but are still intact they are disposed of without harm.   The indications are that kept human cells could be profitably employed in the same way.

AN EXPERIMENTAL STUDY OF PAROTITIS (MUMPS).

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PLATES 53 TO 55.

Studies dealing with the etiology of mumps have been comparatively few in number, and fall into two groups. In the earlier investigations, made chiefly in France,[1] but also in Germany[2] and in America[3] ordinary bacteriological methods were employed, and cocci were isolated from the blood, saliva, and fluid aspirated from the swollen parotid glands.

Attempts to produce parotitis in animals by the inoculation of these cocci proved entirely unsuccessful and the negative results of Roux and Pasteur,[4] and Fichera,[5] who failed to find the cocci demonstrated by other observers, further show that these early studies did not solve the problem of the etiology of epidemic parotitis. The studies made since 1908, when Granata[6] attacked the subject from a new point of view by using the filtrate of patients' saliva for animal inoculation, laid more stress on the reproduction of the disease in laboratory animals than on the isolation of a bacterium from human cases. Granata was the first to conclude that the virus of parotitis may be filterable, basing his conclusions upon the results obtained by inoculating the sterile saliva filtrate from two patients into the

[1] Charrin and Capitan, cited by Védrènes, *Mem. de méd. de chir. et de pharm. mil.*, 1882, xxxviii, 167. Laveran and Catrin, *Compt. rend. Soc. de biol.*, 1893, v, 528. Busquet, *Rev. de méd.*, 1896, xvi, 744. Tessier, P., and Esmein, C., *Compt. rend. Soc. de biol.*, 1906, lviii, pt. 1, 803, 853.

[2] Bien and Michaelis, M., *Verhandl. d. XV Cong. f. inn. Med.*, 1897, xv, 441.

[3] Mecray and Walsh, *Med. Rec.*, 1896, l, 440.

[4] Roux and Pasteur, cited by Védrènes, *Mem. de méd. de chir. et de pharm. mil.*, 1882, xxxviii, 167.

[5] Fichera, G., *Bull. d. r. Accad. di Roma*, 1905, xxxi, 29.

[6] Granata, S., *Med. ital.*, 1908, vi, 647, 672.

blood, parotid gland, and subcutaneous tissue of rabbits. A rise of temperature lasting three days followed the intravenous injections, and swelling of the parotid gland one to two weeks in duration resulted from the other inoculations.

Gordon[7] also used a filtrate of the saliva for the intracerebral inoculation of monkeys. Four of the animals died, having developed meningeal symptoms on the fourth day. At autopsy a lymphocytic meningitis was found with marked degenerative changes in the neurons of the cerebral cortex and anterior horns of the cord. Cultures from the meninges remained sterile. One monkey, inoculated intraperitoneally and intravenously, became ill on the eleventh day and showed swelling of the parotids and stiffness of both jaws, but without nervous symptoms. Recovery was complete. Attempts to transfer the "disease" from one monkey to another with filtrates of the brain and cord from the fatal cases proved entirely unsuccessful. The monkey which survived the longest showed an interstitial parotitis and an interstitial hepatitis, as well. Gordon concludes that mumps is due to a filterable virus of comparatively low virulence.

The work of Nicolle and Conseil[8] is interesting because of a mononuclear leukocytosis detected in one of three monkeys into whose parotid glands they had injected fluid aspirated from the parotid glands of children ill with mumps. After an incubation stage of sixteen to twenty-six days, the animals developed fever lasting two to seven days, and the parotid became swollen in one monkey. No bacteria were demonstrable in the fluid obtained from the human parotid glands.

It will be seen that the experiments of Granata and of Nicolle and Conseil give only suggestive, not entirely successful results so far as the reproduction of mumps in animals is concerned, while Gordon's intracerebral inoculations are chiefly interesting in view of the nervous symptoms sometimes exhibited by patients suffering from epidemic parotitis.

[7] Gordon, M. H., *Reports to the Local Government Board on Public Health and Medical Subjects,* London, 1914, N. s., No. 96.

[8] Nicolle, C., and Conseil, E., *Compt. rend. Acad. d. sc.,* 1913, clvii, 340.

EXPERIMENTAL PART.

Intratesticular inoculation of laboratory animals with infected materials has proved an aid to bacteriological research. The testis acts as an enriching medium, making cultural studies more possible. In addition, the pathological changes caused by the growth of the inoculated virus and the clinical symptoms resulting from such changes can be observed. The fact that orchitis is a frequent complication of mumps in human beings suggested that the animals used in an experimental study of the disease should be inoculated into the testis as well as into the parotid. Cultures from the inoculated organs could then be made according to Noguchi's anaerobic method, while the direct results of the inoculations could be studied. The latter only will be given in this paper.

Cats, rabbits, and monkeys were employed, but as neither rabbits nor monkeys gave promising results, the work was for the time being continued with cats alone.

We are indebted to Dr. Leopold F. Haas and Miss Gillaume, through whose courtesy material for study was obtained from children at the Home for the Friendless; to Dr. Alfred F. Hess and Dr. Sophie Rabinoff, who placed material at the Hebrew Infant Asylum at our disposal; and also to Dr. Matthias Nicoll, Jr., who sent us cases from the Department of Health of the City of New York. The method of obtaining material was uniform. Patients with swollen parotids and symptoms of one to three days' duration were asked to rinse the mouth with sterile salt solution and expectorate into a sterile glass dish. Whenever advisable the parotids were gently massaged at the same time. The secretion thus obtained was filtered through a new Berkefeld candle, and the clear filtrate tested for sterility by aerobic and anaerobic methods. It was found to be uniformly sterile.

The cats were anesthetized with ether, and the skin over the parotid region denuded of hair, then carefully cleansed. With a new, sharp needle, it was not difficult to enter the parotid gland and inject 1 cc. of fluid. Very large, old cats were not favorable because they have a thick layer of fat and dense connective tissue over the gland, making it difficult of access. The fluid is more apt to be injected into

this tissue than into the parotid and the resulting periglandular swelling obscures the results. Well grown young cats proved suitable. The testicles were prepared in the same way as the parotid, and no difficulty was encountered in injecting 1 cc. of fluid.

Before anesthesia the temperature of the cats was recorded, and an actual as well as a differential leukocyte count made. These tests were repeated daily throughout the period of observation.

The animals recovered rapidly from the ether and showed no ill effects from the inoculations. The following day the temperature had usually risen 0.5° C., but the white blood cells had not increased in number. There was, as a rule, slight tenderness in the inoculated testicle, and often in the parotid as well. This was evidently of mechanical and not of inflammatory origin, and always disappeared within another twenty-four hours, leaving the cats apparently well on the second day, though the temperature was 0.5 to 0.8° C. above normal. After six or seven days, tenderness returned in the testis, accompanied by swelling; and similar symptoms appeared in the parotid An increase in the leukocytes became apparent two days after inoculation, and reached the maximum in about seven to fourteen days, coinciding with the height of the fever. The swelling and pain in the parotid lasted two to five days, but the testicular swelling rarely subsided in less than ten to fourteen days. In the third week all the symptoms began to disappear, the leukocytes reaching the normal first, the tenderness disappearing at the same time, and the fever persisting for another week. While tenderness on palpation of the parotids was less marked than that of the testes, and the swelling never reached the stage of marked facial asymmetry, the cats nevertheless manifested some degree of discomfort in the inoculated parotid. The appetite was only slightly affected, and at no period of the experiments did the cats seem especially ill. The disease was not fatal in any instance. The following protocol is typical.

Cat A.—Inoculated Jan. 23, 1915, with sterile filtrate made from the saliva of four children who had been ill two days. 1 cc. injected into left parotid and into left testicle, respectively (Protocol I, Text-fig. 1).

PROTOCOL I.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
Jan. 23	36.4	8,400	Polynuclears 60; large mononuclears 19; small mononuclears 20; eosinophils 2.
" 24	37.0	8,800	Animal seems well. Left testis slightly tender.
" 25	37.2	9,000	No signs.
" 26	37.6	. . . .	
" 27	37.7	9,600	No signs. Polynuclears 71; large mononuclears 14; small mononuclears 14; eosinophils 1.
" 29	37.6	14,400	No signs. Polynuclears 73; large mononuclears 14; small mononuclears 13; eosinophils 3.
" 30	37.8	24,000	Left testis quite tender and slightly swollen. Parotid tender. Polynuclears 70; large mono-nuclears 14; small mononuclears 13; eosino-phils 1.
Feb. 1	38.2	23,000	Polynuclears 75; large mononuclears 8; small mononuclears 16; eosinophils 1. Signs un-changed.
" 3	38.0	29,000	Polynuclears 64; large mononuclears 16; small mononuclears 19; eosinophils 1. Left testis very sensitive and swollen. Parotid signs have disappeared.
" 5	38.0	27,000	Signs the same.
" 6	39.2	32,000	Polynuclears 64; large mononuclears 13; small mononuclears 22; eosinophils 1. Tenderness very marked.
" 8	39.4	31,000	No change.
" 9	38.8	28,000	" "
" 11 ·	38.7	. . . .	
" 13	38.9	12,000	Tenderness less marked.
" 15	38.5	10,000	No tenderness. Left testis slightly smaller than right.
" 16	38.0	. . . .	Chloroformed.

Left testis smaller; on section it is grey and cloudy, but scarcely firmer than the right; neither hemorrhages nor other lesions apparent. Left parotid no larger than right; on section nothing obvious. Microscopically foci of cellular infiltration are found in both parotid and testis, and epithelial degeneration in the latter. The uninoculated glands on the right side are quite normal.

Cat A. Inoculated Jan.23,1915. 1ᵃ Inoculation of Virus.
1 cc of Sterile Saliva Filtrate Into Left Parotid.
1 cc of Sterile Saliva Filtrate Into Left Testicle.

	Jan 23 AM PM	24 AM PM	25 AM PM	26 AM PM	27 AM PM	29 AM PM	30 AM PM	Feb.1 AM PM	3 AM PM	5 AM PM	6 AM PM	8 AM PM	9 AM PM	11 AM PM	13 AM PM	15 AM PM	16 AM PM
39°																	
38°																	
37°																	Killed
36°																	
35°																	
34°																	
Leuko-cytes	8400	8800	9000	9600	14400	18000	24000	23000	29000	27000	32000	31000	28000		12000	10000	
Tender-ness								Testis & Parotid Tender & Swollen.	Parotid Well. Testis Very Tender.	Very Tender.	Unchanged.				Signs Less Marked.	No Signs.	

TEXT-FIG. 1.

Transmission from Cat to Cat.

At intervals varying from seven days to four weeks, the animals were etherized, the inoculated glands removed with aseptic precautions, and extracts or emulsions from them injected into other cats. The extracts were prepared in the following way: The organs were cut into small pieces, thoroughly ground up in a mortar with sterile sand and salt solution in a proportion of about 1 to 10, shaken for 2 to 3 hours at 37° C., and centrifugalized. The opalescent fluid was used for injection after it had been tested for sterility. Emulsions were made by grinding the organs in a small tissue-grinding machine through whose fine wire screen 0.2 to 0.3 cc. of finely divided material passed. This was suspended in 1 to 2 cc. of salt solution and its sterility tested before use. The best time for the reinoculation was found to be from the fourteenth to the seventeenth day when the testicular swelling, the leukocytosis, and the fever were highest. During the first seven to ten days transfers were less uniformly successful. Evidently the reaction requires about two weeks for its maximum development. Atrophy of the inoculated testicle occurred in several cats, after the acute symptoms had abated.

After the third or fourth transfers the reaction developed more rapidly and the effects were more severe. But after the sixth or seventh transfers the reaction perceptibly declined. In one instance only were effects observed at the eighth transfer.

Certain illustrative experiments are given in the text-figures and protocols which follow.

Cat B.—Fourth transfer of Strain III. Inoculated Feb. 6, 1915, with sterile extracts of parotid and testis from Cat K (Protocol II, Text-fig. 2).

Left parotid injected with sterile extract from left parotid.

" testis " " " " " " "

Right " " " " " " " testis.

PROTOCOL II.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
Feb. 6	38.0	15,000	Polynuclears 62; large mononuclears 17; small mononuclears 19; eosinophils 2. No signs.
" 7	38.3	16,000	
" 8	38.5	20,000	Polynuclears 70; large mononuclears 16; small mononuclears 16; eosinophils 4. No signs.
" 9	39.1	....	
" 10	39.8	22,000	
" 11	39.9	23,000	Polynuclears 76; large mononuclears 11; small mononuclears 11; eosinophils 2. Left testis tender; right less so.
" 13	39.7	21,000	Polynuclears 75; large mononuclears 13; small mononuclears 8; eosinophils 4. Left testis very tender and enlarged.
" 15	39.5	20,000	
" 16	39.0	....	Chloroformed.

Left testis larger than right; on section both are cloudy and moist compared with those from a normal cat. The left parotid measures 3.5×2.5 cm., while the right is 3×2.5 cm. in diameter. The left is pinkish in color and more moist and granular on section. Microscopically the right testis shows a more marked degree of "spermatorrhexis" than the left.
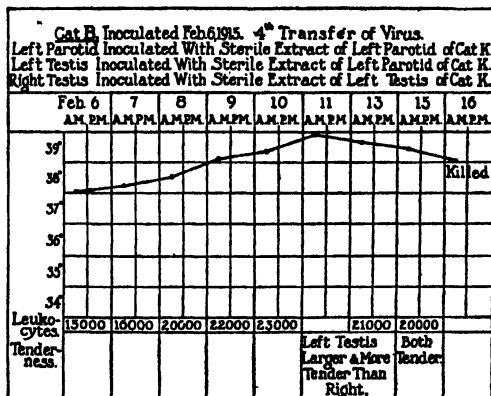
Text-Fig. 2.

Cat C.—Third transfer of Strain IV. Inoculated Mar. 11, 1915, with sterile extracts of parotid and testis from Cat J (Protocol III, Text-fig. 3).

Left parotid injected with extract from left parotid.

" testis " " " " " "

Right " " " " " " testis.

PROTOCOL III.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
Mar. 11	37.0	16,000	Polynuclears 62; large mononuclears 16, small mononuclears 18; eosinophils 4.
" 12	37.3	17,000	Testes slightly sensitive.
" 13	37.4	17,500	No signs.
" 14	37.8	. . . .	" "
" 15	38.8	20,000	Very slight tenderness.
" 16	39.0	24,000	Polynuclears 77; large mononuclears 11; small mononuclears 9; eosinophils 3. Swelling and tenderness in both testes.
" 17	39.2	23,000	Signs the same. Parotid not sensitive.
" 18	39.6	22,000	Polynuclears 79; large mononuclears 6; small mononuclears 10; eosinophils 5. Signs unchanged.
" 19	39.4	21,000	
" 20	39.2	18,000	Polynuclears 70; large mononuclears 4; small mononuclears 18; eosinophils 8. Signs unchanged.
" 22	38.8	15,000	Tenderness less
" 23	38.6	. . . .	Chloroformed.

Testes scarcely enlarged, but left one, on section, is firmer than the right; both are cloudy. Left parotid is pinkish in color but not larger than the right. Microscopically both testes show marked epithelial degeneration, and, in addition, the left is the seat of cellular infiltration (Fig. 3). Cultures sterile.
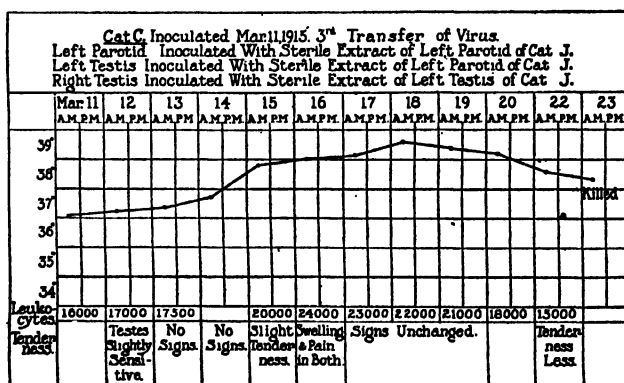
TEXT-FIG 3.

Control Experiments with Normal Organs.

As control experiments, extracts were prepared from the parotid glands and testicles of normal cats, and injected into three healthy cats in doses equal to those previously used. Neither enlargement nor tenderness developed in the glands inoculated in this way nor did the rise in temperature last longer than two days. Coincident with the temperature rise there was a polynuclear leukocytosis of two days' duration. At autopsy no macroscopic or microscopic changes were demonstrable in either the parotids or the testicles, while active spermatogenesis was in progress.

Cat D.—Inoculated Mar. 4, 1915, with sterile extract of parotid and testis from a normal cat (Protocol IV, Text-fig. 4).

Left parotid injected with sterile extract from left parotid.
" testis " " " " " " testis.

PROTOCOL IV.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Mar. 4	38.3	14,000	Polynuclears 65; large mononuclears 12; small mononuclears 20; eosinophils 3.
" 5	38.6	14,500	Left testis slightly tender.
" 6	38.8	....	No signs.
" 8	38.6	12,000	Polynuclears 69; large mononuclears 13; small mononuclears 7; eosinophils 1. No signs.
" 9	38.8	....	
" 10	38.4	20,000	Polynuclears 65; large mononuclears 10; small mononuclears 12; eosinophils 3. No signs.
" 11	38.9	....	
" 12	39.4	21,000	Polynuclears 84; large mononuclears 8; small mononuclears 4; eosinophils 4. No signs.
" 13	39.0	18,000	Polynuclears 71; large mononuclears 20; small mononuclears 6; eosinophils 3. No signs.
" 15	38.8	16,000	Polynuclears 64; large mononuclears 28; small mononuclears 6; eosinophils 2. No signs.
" 16	38.8	15,000	Polynuclears 66; large mononuclears 14; small mononuclears 18; eosinophils 2. No signs.
" 17	38.5	....	Chloroformed.

No difference observed in the parotids and testes on the two sides, on macroscopic and on microscopic examination. Spermatogenesis appeared normal.

Cat D. Inoculated Mar.4,1915 With Sterile Extracts From Testes & Parotids of a Normal Cat. Left Parotid With Sterile Extract From Normal Parotids. Left Testis With Sterile Extract From Normal Testes.																							
	Mar.4		5		6		8		9		10		11		12		13		15		16		17
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
39																								
38																							Killed	
37																								
36																								
35																								
34																								
Leukocytes.	14000		14500		12000		12000				20000				21000		18000		16000		15000			
Tenderness.	Left Testis Slightly Tender		No Signs In Parotids Or In Testicles.														No Signs.							

TEXT-FIG. 4.

Control Experiments with Normal Saliva.

Other control experiments were made by injecting the sterile filtrates of normal saliva obtained from three healthy persons into the parotid glands and testes of normal cats. The resulting rise in temperature was not greater than 0.5° C. While the leukocytes were only increased by two or three thousand cells, the polymorphonuclear cells were relatively more numerous after the injection, and they did not regain the normal level during the period of observation which lasted from twelve to sixteen days. The small mononuclear cells were relatively diminished. Neither swelling nor tenderness developed in the inoculated glands, and even after the third and fourth successive transfer with this material no macroscopic lesions were discernable at autopsy. On microscopical examination the parotid glands showed no difference between the inoculated and the uninoculated side, and the same was true of the testes in seven of the animals. In two cats, however, the inoculated testis showed a moderate amount of degenerative change in the tubular epithelium. The lesion, however, was not comparable to that in the testes of the cats inoculated with saliva from mumps patients, being far less marked in extent and in degree. It should simply be mentioned that in two instances, the testes of cats into which saliva filtrates from healthy persons had been

injected were not absolutely normal on microscopic examination after death. Protocol V is typical.

Cat E.—Inoculated Oct. 8 with sterile filtrate of normal saliva into left testis and right parotid gland (Protocol V).

PROTOCOL V.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
Oct. 8	38.6	30,000	Polynuclears 50; large mononuclears 8; small mononuclears 35; eosinophils 7. Slight tenderness of left testis.
" 9	38.3	32,000	Polynuclears 67; large mononuclears 8; small mononuclears 15; eosinophils 10. No tenderness of testis or parotid.
" 11	38.7	33,000	Polynuclears 61; large mononuclears 7; small mononuclears 27; eosinophils 5.
" 13	39.0	29,000	Polynuclears 63; large mononuclears 7; small mononuclears 18; eosinophils 12.
" 14	38.8	32,000	Polynuclears 73; large mononuclears 3; small mononuclears 20; eosinophils 4.
" 15	39.0	30,000	Polynuclears 21; large mononuclears 5; small mononuclears 18; eosinophils 6.
" 16	38.8	25,000	Polynuclears 66; large mononuclears 4; small mononuclears 20; eosinophils 10.
" 18	38.5	22,000	Polynuclears 76; large mononuclears 5; small mononuclears 10; eosinophils 9.
" 19	39.0	32,000	Polynuclears 77; large mononuclears 4; small mononuclears 13; eosinophils 6.
" 20	39.0	30,000	Polynuclears 65; large mononuclears 10; small mononuclears 22; eosinophils 3. No tenderness.
" 21	38.2	....	Chloroformed.

At autopsy there was no difference in the size, color, or consistency of testes or parotid glands.

Microscopically no lesion was found in the inoculated testis or parotid.

Finally sterile salt solution alone was injected into the parotid gland and testes of three cats. The temperature rose only half a degree, and neither microscopic nor macroscopic lesions developed.

Pathology.

1. Gross Appearance. (*a*) *Parotid Gland.*—On exposing the parotid glands in the recently killed cats, the deeper pink color of the inoculated gland was very striking, as was also its larger size. The difference in weight varied from 70 to 340 mg. The inoculated gland was more moist than the uninoculated, and showed on section a granular appearance, due to swelling of the acini. No other changes occurred. The adjacent lymph nodes were usually congested but not distinctly enlarged (Fig. 5). The molar gland, situated just beneath the skin at the angle of the mouth and extending along the lower lip, was uniformly congested and swollen on the inoculated side. During life the buccal mucosa over the gland was distinctly reddened. This symptom could be found in the cat's mouth in four or five days after inoculation and in a few instances was accompanied by swelling. The opening of the duct leading from the injected parotid was always surrounded by a small zone of congestion.

(*b*) *Testis.*—The inoculated testis was larger than the uninoculated, but unchanged in color. On section the cut surface was more gray, cloudy, and moist. In a few instances the point of inoculation was visible as a small dark spot, but otherwise no focal changes were noted.

2. Microscopic Appearance. (*a*) *Parotid.*—The histological changes when present in the parotid were not constant for all the transfers of every strain. In the first transfer they were usually inconspicuous; and at most congestion of the vessels and swelling of the epithelium of the acini were made out, with edema of the interlobular connective tissue.

The most marked changes appeared coincidently with the third and fourth transfers. In some examples, the glands showed infiltration of the interlobular connective tissue with mononuclear and a few polynuclear cells in addition to the edema   The infiltration was most intense about the secretory ducts (Fig. 1) which were sometimes dilated (Fig. 2). The epithelium of the acini was swollen and cloudy in these instances. The areas of · cellular infiltration were more pronounced in some parts of the gland than in others, were always multiple, and could be easily differentiated from the small

lymph nodes which are normally present between the lobules. In some instances infiltration did not appear at all.

(b) *Testis.*—The histological changes in the testis were more constant than were those in the parotid, and, in further contrast, they were more pronounced in the epithelium than in the supporting framework of the gland. The layer of cells next to the basement membrane of the tubules tended to be normal in appearance, but the rest of the cells were often the seat of a change resulting in a diminution in the number of spermatocytes and consequently of the mitotic nuclei normally found. The spermatids were even more altered, showing as the remains of their nuclei irregular and deeply staining granules, while the cell bodies stained poorly or had undergone extensive lysis. In these cases, spermatozoa, as would be expected, were diminished in number and few were normal in appearance, their broken-up condition being easily discernible (Fig. 3). This process of imperfect spermatogenesis and of disintegration or spermatorrhexis was further indicated by the empty condition of the tubules of the rete and epididymis. Epithelial changes of some degree were present in some part of practically all the inoculated testicles. Interstitial cellular infiltration, on the other hand, occurred far less frequently in the testicle than in the parotid gland. In a few instances it was pronounced about groups of tubules, whose epithelium showed marked degeneration (Fig. 4). Another striking change in the testis was noted in the interstitial cells. These were larger than normal, and, in several instances, actually increased in number (Fig. 3), forming large masses between the tubules.

Fibrous connective tissue in increased amount with a diminution in the size and number of the convoluted tubules was found in the atrophic testes.

The microscopic examinations were always made with material removed from animals killed with chloroform and fixed at once in Zenker's fluid.

Reinoculation Experiments.

Four cats which reacted well were allowed to survive until all symptoms abated and were then reinjected with filtrates prepared from fresh cases of mumps in the manner already described (page

358). As a result of the second injection the temperature rose only 0.8° C., and the leukocytes increased only by 5,000 cells, whereas after the first injection the rise of temperature was about 1.9° C. above normal, and the white cells had more than doubled. No sensitiveness of either parotid or testis developed after the second injection. This result suggested that the first injection had reduced considerably the reaction to the second. Whether the effect was one of immunization can only be conjectured.

Cat F.—Inoculated Jan. 15, 1915, with sterile extract of parotid and testis from Cat I, making this cat the third transfer of this series (Protocol VI, Text-fig. 5).

Left parotid injected with left parotid of Cat I.
" testis " " " testis " " "
Right " " " " parotid " " "

PROTOCOL VI.

Date.	Temperature.	Leukocytes.	Remarks.
1915	*°C.*		
Jan. 15	37.0	9,000	Polynuclears 66; large mononuclears 17; small mononuclears 12; eosinophils 5.
" 22	38.0	20,000	Polynuclears 80; large mononuclears 15; small mononuclears 5; eosinophils 0. Left parotid tender and swollen; both testes tender.
" 25	38.4	24,000	Parotid swelling gone. Both testes tenaer and enlarged.
" 30	39.0	23,000	Signs unchanged.
Feb. 5	39.0	19,000	Tenderness of testes slight. Polynuclears 60; large mononuclears 14; small mononuclears 26; eosinophils 0.
" 10	38.3	15,000	No signs. Polynuclears 62; large mononuclears 15; small mononuclears 20; eosinophils 3.
" 19	37.2	10,000	No signs. Reinoculated into left testis and left parotid with 1 cc. of sterile filtrate from 4 fresh cases of mumps.
" 20	37.6	12,000	Polynuclears 68; large mononuclears 15; small mononuclears 15; eosinophils 2. No signs.
" 23	37.8	15,000	Polynuclears 72; large mononuclears 12; small mononuclears 13; eosinophils 3.
" 27	38.0	12,000	Polynuclears 70; large mononuclears 13; small mononuclears 15; eosinophils 2. No signs.
Mar. 1	37.5	12,000	No signs.
" 9	37.2	10,000	" " Chloroformed.
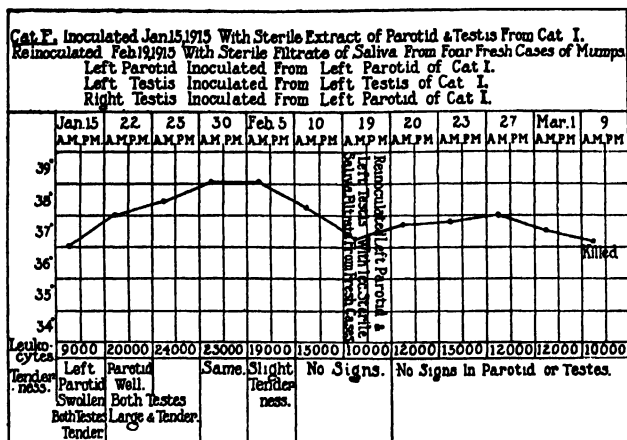
TEXT-FIG. 5.

Serum Protection Experiments.

In order to test the protective power of the serum of a cat which had entirely recovered from an inoculation with the filtrate three months before, three experiments were made. Material from the enlarged glands of cats killed at the height of the reaction had been kept in glycerine in the ice box for four months. This was emulsified in the usual way and the emulsion was left in contact with the serum for two hours at a temperature of 37° C. The mixture was then injected into the parotid gland and testicle of normal cats, control animals being inoculated at the same time with glycerinated material which had not been treated with the serum. A definite difference in the effects was noted in the two sets of animals. The animals receiving the emulsion-serum mixture showed less reaction than those receiving the emulsion alone. Glycerination of the testicles and parotids seemed to make little change, within the period of the experiment, in the results of the inoculation.

The protocols and text-figures illustrating the experiments follow.

1. Control.

Cat G.—Inoculated May 14, 1915, with glycerinated material from Cat K, killed Feb. 6 (Protocol VII, Text-fig. 6).

Left parotid inoculated with left parotid of Cat K.
" testis " " " testis " " "

PROTOCOL VII.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
May 14	38.4	15,000	Polynuclears 68; large mononuclears 10; small mononuclears 14; eosinophils 8.
" 15	38.4	....	No signs.
" 17	38.8	18,000	" " Polynuclears 78; large mononuclears 7; small mononuclears 12; eosinophils 3.
" 20	39.1	30,000	No signs. Polynuclears 70; large mononuclears 13; small mononuclears 8; eosinophils 9.
" 22	40.0	36,000	Left testis sensitive.
" 24	39.8	24,000	" " " Polynuclears 59; large mononuclears 14; small mononuclears 20; eosinophils 7.
" 28	39.0	26,000	Left testis sensitive. Polynuclears 65; large mononuclears 12; small mononuclears 17; eosinophils 6.
June 2	38.3	20,000	No signs. Polynuclears 67; large mononuclear 9; small mononuclears 21; eosinophils 3.
" 8	38.5	18,000	No signs.

Animal survived.

Cat G. Control For H. (Chart 7.)
Inoculated May 14 With Glycerinated Virus From Cat K. Killed Feb 6, 1915.
Left Parotid Inoculated With Left Parotid.
Left Testis Inoculated With Left Testis.
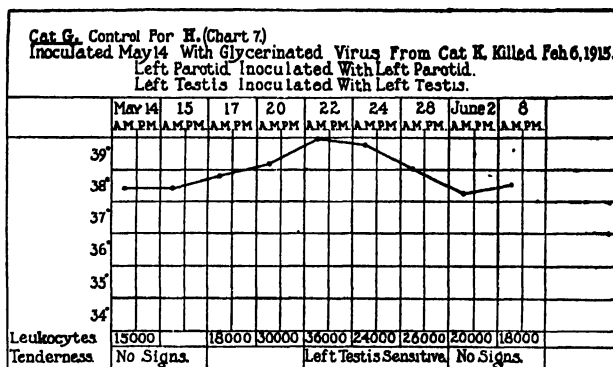
TEXT-FIG. 6.

2. Serum Experiment.

Cat H.—Inoculated May 14, 1915, with glycerinated material from Cat K, kept in contact with serum of Cat L for two hours at 37°C. (Protocol VIII, Text-fig. 7).   Cat L was inoculated Feb. 6; well since Mar. 1; bled on May 12.

Left parotid inoculated with left parotid of Cat K.
" testis   "   "   " testis   "   "   "

PROTOCOL VIII.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
May 14	38.3	16,000	Polynuclears 70; large mononuclears 12; small mononuclears 12; eosinophils 4.
" 15	38.2	17,000	No signs.
" 17	38.9	19,000	Polynuclears 74; large mononuclears 10; small mononuclears 13; eosinophils 3. No signs.
" 18	38.9	....	
" 19	39.0	20,000	Polynuclears 69; large mononuclears 13; small mononuclears 14; eosinophils 4. No signs.
" 20	39.0	....	Left testis slightly tender.
" 21	38.6	18,000	No signs.
" 22	38.2	18,000	Polynuclears 71; large mononuclears 14; small mononuclears 13; eosinophils 2. No signs.
" 24	38.0	....	No signs.
June 4	38.0	15,000	" "

Chloroformed. Neither parotid nor testis showed any lesion on gross or microscopic examination.
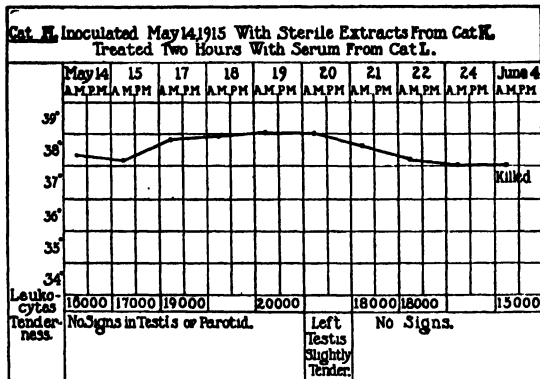
TEXT-FIG. 7.

In comparing the case histories of the two cats, it will be noted that there are differences in the temperature and leukocyte counts, as well as in the reaction within the organs. Thus while the temperature of the control animal rose to 40° C., and remained above 39° for a week, the cat injected with the serum-treated material showed a rise to 39° C. over a period of only two days. Again, the

white cells of the control had increased to double their initial number on the eighth day after inoculation, and had not returned to normal ten days later; in the serum cat, on the contrary, they had risen only 4,000 in actual number by the fifth day, and the count had become practically normal within a week. The differential count remained quite unchanged in the second animal, while the control showed the early characteristic polynuclear cytosis and the later lymphocytosis.

The serum from a normal cat which had not been inoculated was employed in like manner to the immune serum, to serve as a serum control. The normal serum did not inhibit the action of the virus, as Protocol IX indicates.

Cat M.—Inoculated Dec. 15 with sterile extract of parotid and testis of an inoculated cat, which had been kept in contact with normal cat serum for 2 hours at 37°C. Right parotid and left testis injected.

PROTOCOL IX.

Date.	Tempera-ture.	Leukocytes.	Remarks.
1915	*°C.*		
Dec. 15	38.9	21,000	Polynuclears 62; large mononuclears 19; small mononuclears 14; eosinophils 5.
" 16	38.9	23,000	No signs.
" 17	39.3	. . . .	
" 18	39.2	25,000	Polynuclears 65; large mononuclears 21; small mononuclears 12; eosinophils 1.
" 20	39.3	30,000	Left testis tender.
" 21	39.2	. . . .	" " more tender.
" 22	39.5	32,000	
" 23	39.7	. . . .	" " tender.
" 24	39.6	35,000	" " "
			Polynuclears 56; large mononuclears 18; small mononuclears 21; eosinophils 5. Chloroformed.

At autopsy the right parotid was distinctly swollen, and more congested than the left. The left testis was slightly larger than the right.

These experiments indicate that the serum of the cats employed has the power to reduce the development of the reaction produced by the injection of testicular and parotid materials derived from cats treated with filtrates of the saliva from cases of acute parotitis.

Blood Changes.

Daily blood counts were made on several normal cats, to ascertain the blood picture in healthy animals. It was found that the average number of leukocytes varied from 16,000 to 20,000, and that a difference of 5,000 from day to day was not unusual. It follows, therefore, that only a very decided increase in the number of white cells can have any significance in the cat.

The white cells began to increase within the first forty-eight hours after the inoculations, while on the seventh or eighth day a sudden additional rise, often doubling the initial count, may occur. The increase is maintained, with daily fluctuations, for a period of eight to ten days, the maximum being reached on about the fourteenth to the sixteenth day. The fall in number takes place gradually, the initial number being reached as a rule in about four weeks. During the first two weeks the leukocytosis is polymorphonuclear in character. At the end of the second or beginning of the third week, when the fever and glandular swellings are at the highest, a lymphocytosis is noted. The large mononuclear and eosinophil cells remained practically unchanged throughout.

The blood picture as described in the experiments is not very dissimilar from the one observed in cases of parotitis in man with testicular complications. Wile[9] has noted the change from a lymphocytosis to a polynuclear leukocytosis under these conditions. Apparently the change is inconstant, since Feiling[10] has not always found it. Recently Aubertin and Chabanier[11] have noted an initial polynuclear leukocytosis in all cases of mumps examined, the lymphocytosis following later, a fact which does not agree with Feiling's observations, which are to the effect that a relative and absolute lymphocytosis is present on the first day of epidemic parotitis. The increase in mononuclear cells found by Nicolle and Conseil in one of their monkeys, which had been inoculated into the parotid but not into the testicle, is of some interest in this connection, as is the polynuclear leukocytosis without the later appearance of a lymphocytosis

[9] Wile, I. S., *Arch. Pediat.*, 1906, xxii, 669.

[10] Feiling, A., *Quart. Jour. Med.*, 1915, viii, 255.

[11] Aubertin, Ch., and Chabanier, H., *Arch. d. mal. du coeur*, 1915, viii, 1.

noted in cats inoculated intratesticularly with material from normal cats.

That the polynuclear leukocytosis is not due to suppurative inflammation of the testes is indicated by the microscopic examinations, which showed also that at the time the testicles were removed no excess of polynuclear cells occurred in them.

DISCUSSION.

The object of the study described in the preceding pages was the reproduction of the chief organic lesions of parotitis in animals by means of filtered extracts of the saliva derived from persons suffering from acute parotitis. The experiments indicate that certain definite results have been obtained. It now appears that the injections into cats of bacteria-free filtrates of the saliva derived from cases of acute parotitis are capable of setting up a series of pathological changes in the parotids and testicles, expressed by fever, leukocytosis, tenderness, and swelling attended by definite histological alterations. Whether these changes accurately reproduce the condition arising in man in acute parotitis need not now be decided. The interest of the observations increases, however, in view of the fact that the effect of the injections is intensified by successive transfers of the inoculated organs from animal to animal through several passages.

Upon what principle present in the saliva· the effects produced depend need not be discussed at the moment. It is obvious that ordinary bacteria can be excluded, since they were never found in microscopic preparations of the filtrate, or in aerobic or anaerobic (tissue) cultures, or in film preparations and sections made from the inoculated parotids and testes. On the other hand, it seems necessary to suppose that the active agent is of living nature; and thus the conclusion of a minute filterable virus is suggested.

The effects attributed to the saliva from cases of parotitis do not follow the injection of filtrates prepared with the saliva of normal persons. Moreover, and this point is a suggestive one, the active agent present in the saliva in cases of parotitis is neutralized or rendered ineffective by the serum of a cat which had been permitted to survive the injection of testicular and parotid emulsions, while the serum of a

normal cat had no such power. Assuming the activity of the salivary filtrate to be due to a living virus the power of prevention exercised by the serum may be assumed to be due to an immune body which was developed in the inoculated cat.

SUMMARY AND CONCLUSION.

Cats injected into the parotid gland and testicle with a bacterial sterile filtrate of the salivary secretion of children in the active stage of parotitis or mumps can be made to develop a pathological condition having several points of resemblance to the condition present in mumps in human beings.

After an incubation stage of from five to eight days definite changes have been noted in the temperature, blood leukocytes, and inoculated organs.

The temperature rise begins within twenty-four hours of the inoculations and reaches a maximum in from seven to fourteen days. The febrile rise fluctuates between 1° and 2.5° C.

The white blood cells begin to increase on the second day following the inoculation. The first change is a polymorphonuclear leukocytosis which precedes the glandular swellings. This initial rise is followed by a decline, after which the lymphocytes increase. The increase is confined to the small lymphocytes, which increase to from 7 to 10 per cent of their initial number.

The inoculated glands become swollen and tender. The swelling and tenderness become apparent from the fifth to the ninth days and persist for a variable period. The parotid changes are less constant or less obvious than are the testicular. The latter are constant and endure from eight to twelve days.

The rise of temperature and the leukocytosis precede the glandular swelling, but all the changes reach the maximum at about the same time, after which they decline gradually. What may be regarded as normal conditions are reestablished in four weeks or less.

The intraparotid and intratesticular injections of extracts of normal parotid gland and testicles may cause a mild rise of temperature and leukocytosis of brief duration, but swelling and tenderness are absent. 'The white cells increased are the polymorphonuclears and not the lymphocytes.

The intraparotid and intratesticular injections of filtrates of normal saliva may cause a mild rise of temperature of very brief duration, but leukocytosis, swelling, and tenderness do not appear.

The histological changes in the parotid gland when present consist chiefly of edema of the interlobular connective tissue with mononuclear interstitial infiltration about the ducts and elsewhere. In cases of long duration the ducts may be dilated. But in some instances the swollen gland while showing congestion and edema in gross showed inconspicuous changes under the microscope. The histological changes in the testicle are of two kinds: inconstant changes of cellular invasion between the tubules and swelling or even multiplication of the interstitial cells, constant ones consisting of degeneration of the epithelium and interference with spermatogenesis, a condition to which we have applied the term "spermatorrhexis."

The pathological conditions set up by the filtrate derived from the salivary secretion of cases of acute parotitis are intensified by successive transfers through a small series of cats of the extract and emulsion of the parotid gland and testicle previously inoculated.

The pathological changes are also prevented or reduced when the extract or emulsion is previously incubated with a quantity of blood serum obtained from a cat which has survived inoculation. Normal serum, on the other hand, has no such inhibiting effect.

The deduction from these experiments is to the effect that the salivary secretion in parotitis or mumps contains a filterable substance capable of setting up a series of definite pathological conditions when inoculated into the testicle and parotid glands of cats. Whether this active material is a microorganism and if so whether it is the specific microbic cause of parotitis or mumps remains to be ascertained.

EXPLANATION OF PLATES.

PLATE 53.

FIG. 1. Parotid gland, showing cellular infiltration around the secretory ducts. Cat killed after 18 days.

FIG. 2. Parotid gland, showing cellular infiltration and dilatation of the secretory ducts. Cat killed after 17 days.

PLATE 54.

FIG. 3. Testicle, showing cellular infiltration around the tubules, and spermatorrhexis. Cat killed after 12 days.

FIG. 4. Testicle, showing increase in the size and number of the interstitial cells, and spermatorrhexis. Cat killed after 5 weeks.

PLATE 55.

FIG. 5. Parotid glands, showing swelling of the inoculated (left) side at autopsy. Cat killed after 13 days.
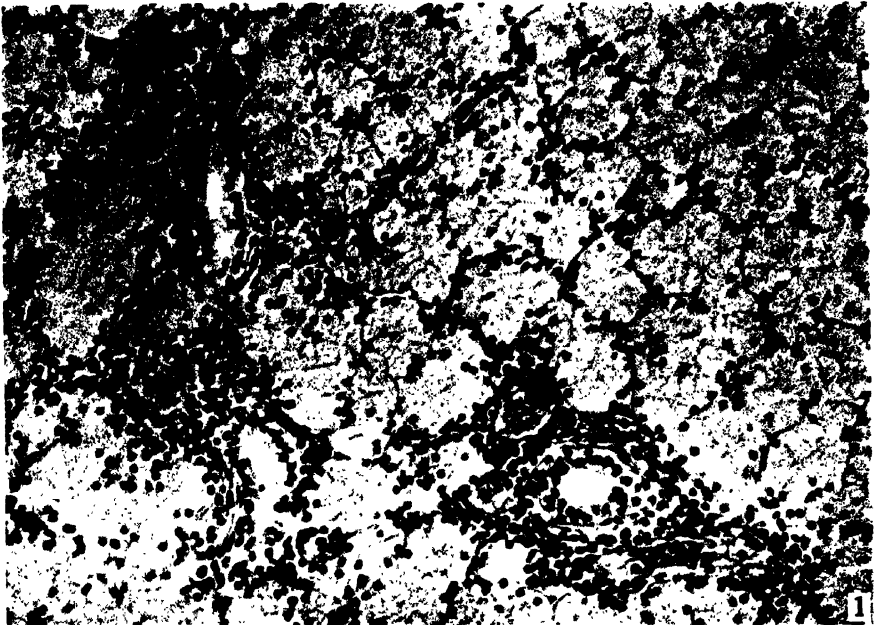
(Wollstein: Experimental Study of Parotitis (Mumps).)
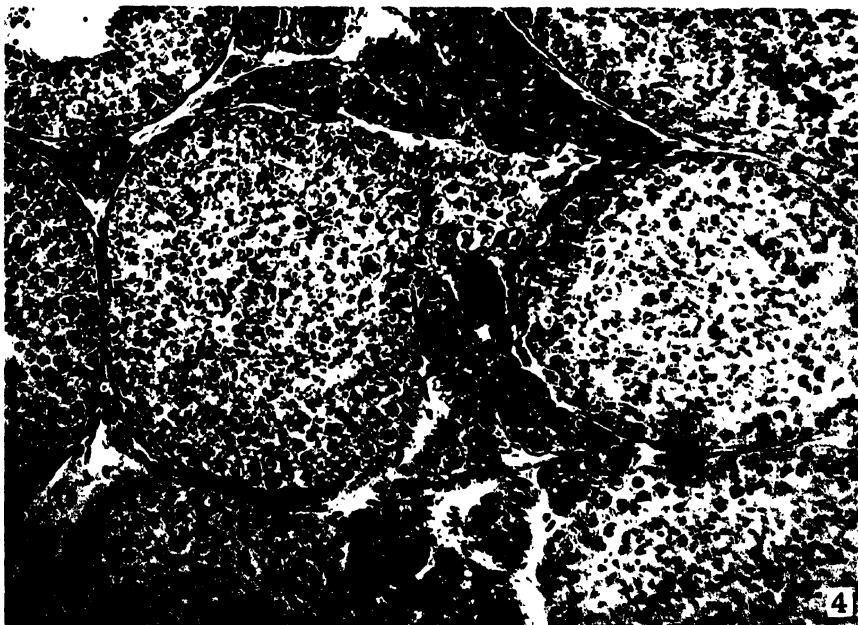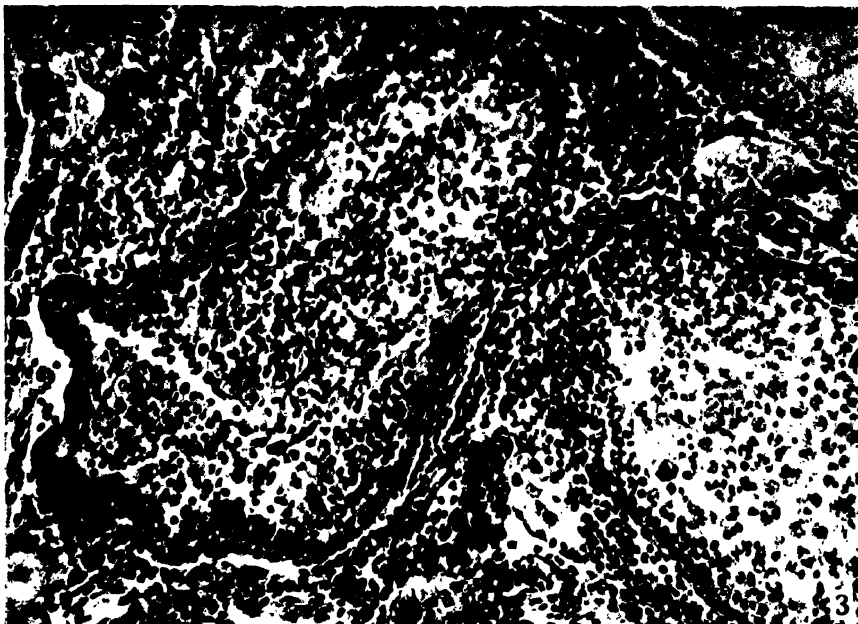
Left parotid, inoculated.   Right parotid, not inoculated.

FIG. 5.

A METHOD FOR THE RAPID PREPARATION OF ANTI-MENINGITIS SERUM.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The European war has brought about a greatly increased demand for antimeningococcic serum. Epidemic meningitis is known to be one of the attendants of armies in barracks and in the field, and the present war has proven no exception to this rule. Information is at hand indicating that at least the principal, if not all the belligerent countries have suffered from epidemics of meningitis to a greater or less extent. Now that we have knowledge of the manner in which epidemic meningitis is conveyed, namely, by means of meningococcus carriers who harbor that microorganism in the nasopharynx, the appearance of meningitis in widely separated countries and places is not at all remarkable. Meningococcic meningitis has prevailed either in epidemic form or sporadically in many European countries during the past 10 or more years. Hence the bringing together of recruits has had the effect of introducing carriers of the infection into the midst of the European armies. Not until the European war is over and the medical data of the war have been collected and analyzed shall we know whether the epidemics have been of equal severity throughout or whether some have been of greater severity than others. But the interim observations and studies have already yielded certain definite information of great importance in respect to the combating of the disease through the employment of the specific antimeningococcus serum.

Types of Meningococcus.

Biological studies of meningococci have led to their separation into two main groups, called respectively meningococci, or normal

meningococci,[1] and parameningococci (Dopter[2]). The two main groups are distinguished not by their cultural properties, but by their immunity reactions. This divergence in immunity reaction has proven of the greatest importance in perfecting the antimeningitis serum, since its action is specific and it affects those types of meningococci only for which its antibodies possess affinity.

At the outset, and before the two main groups of meningococci were clearly differentiated, the antimeningitis serum was prepared by employing a number of strains of meningococci in the immunizing process. The object, of course, was to cover ordinary variation in immunity properties of the different cultures; but with the discovery of the two types of meningococci this haphazard method no longer sufficed and it became necessary to use for purposes of immunization representatives of the two groups now designated meningococci and parameningococci. The sera now being prepared are for the most part produced by immunizing horses either with mixtures of these types of meningococci or with alternate injections of the two types.

Our recent studies indicate, however, that it does not suffice merely to employ representative normal and representative parameningococcus strains for the purpose of immunization, for while the two groups are, immunologically considered, fairly homogeneous, yet within each group there exist organisms which react weakly to the specific immune bodies produced by other strains of the same type. On the other hand, when these weakly reacting strains are themselves inoculated they evoke the formation of specific immune bodies to which they react strongly. Hence it is desirable to employ for immunization not merely one, but rather several strains of each group; and it is further desirable to change or alternate the strains as new ones are obtained which show an imperfect response to the immunity bodies already present.

The existence of different types of meningococcus was indicated early in the course of the serum treatment of meningitis through the observation that in certain cases the meningococci when brought under the influence of the antiserum failed to be affected by it. Since this was the exception, it was assumed that certain strains of meningo-

cocci were resistant, or fast, to the antimeningitis serum. Further studies, as has been mentioned, established the fact that two great groups of meningococci could be distinguished; and our later studies indicated that even within these groups variants occur which are less subject to the action of a polyvalent antimeningitis serum than the majority of strains.

We are at present only imperfectly informed as to the relative prevalence of the different types of meningococci in given foci of epidemic meningitis. The American experiences and apparently the experiences previous to recent studies in the war zone in France and England have indicated that normal meningococci greatly preponderate in cases of epidemic meningitis. It now appears that, in certain localities at least in which epidemic meningitis prevails in France and England among the army, the proportion of cases caused by normal meningococci as contrasted with cases caused by parameningococci may be no higher than 6 : 4 (Ellis,[3] Arkwright[4]).

This consideration has definite bearing on the preparation of antimeningitis serum and emphasizes the importance of proceeding in its preparation in such a manner as to produce quantitative results in which the antibodies for the parameningococci about equal in amount those for the normal strains.

Mode of Preparation of the Serum.

At the first appearance of the present outbreak of epidemic meningitis in Great Britain, The Rockefeller Institute was no longer engaged in the preparation of the antimeningitis serum. Two circumstances led to the resumption of its manufacture. The first was the probability that the epidemics abroad would extend and the demand for the serum would exceed that available from ordinary sources of supply. Moreover, the identification of The Rockefeller Institute with the original production of the serum brought to it urgent requests for serum from several of the countries at war, with which it seemed imperative to comply. Through the assistance rendered by The Rockefeller Foundation, which has been engaged extensively in war

[3] Ellis, A. W. M., *Brit. Med. Jour.*, 1915, ii, 881.
[4] Arkwright, J. A., *Brit. Med. Jour.*, 1915, ii, 885.

relief, funds were placed at the disposal of the Institute covering the cost of production of the serum, so that it could be supplied gratis to those countries from which the demand came.

But there was a second important reason which led to the resumption of the manufacture of the serum. At the time and after the appearance of epidemic meningitis, particularly among the British recruits, the supply of serum available in England was chiefly that prepared commercially. Its use was distinctly disappointing. Realizing that in all probability the failure lay with the samples of serum available, which by reason of some fault of production or preservation were inactive, it seemed desirable to produce a serum the activity of which could be relied upon (Osler, Rolleston[5]).

In a previous communication[6] from the laboratories of the Institute a method was described by means of which the preparation of the antidysenteric serum was greatly abbreviated. The method consists in making injections into the horse of cultures or extracts of dysentery bacilli on 3 successive days, after which a period of rest is permitted. It was ascertained that the immunity response to the bacteria or bacterial products thus injected was far greater than when they were introduced at periods separated from each other by the ordinary intervals of time. Moreover, it was determined that by this rapid method an efficient polyvalent antidysenteric serum could be produced, representing both the non-acid (Shiga) and the acid (Flexner) groups of bacilli. Instead of a period of 8 or 12 months required by the usual method of immunization, an equally strong serum was produced in the short period of 8 or 12 weeks.

The problem presented, therefore, in respect to the antimeningitis serum seemed essentially similar to that encountered with respect to the antidysenteric serum. In the case of the latter, two groups of the bacilli are dealt with: first, a fixed or Shiga group, which yields a soluble toxin; and second, a fluctuating group made up of very slightly divergent types of bacilli which do not yield a soluble toxin. In the case of the meningococci there are also two groups or types, neither being perhaps altogether fixed: the ordinary or normal meningococci

[5] Discussion on the treatment of cerebrospinal meningitis, *Proc. Roy. Soc. Med., Therap. and Pharmacol. Sec.*, 1915, ix, 1.

[6] Flexner, S., and Amoss, H. L., *Jour. Exper. Med.*, 1915, xxi, 515.

which readily undergo autolysis, and the parameningococci undergoing less perfect autolysis, both yielding, however, a toxic product. Hence it could be assumed that by employing a method similar to that worked out for the antidysenteric serum, horses might be rendered immune and made to yield an efficient polyvalent antimeningococcic serum in a period of time far shorter than is required by the usual method of subcutaneous inoculation. The substances employed in both instances are the same; namely, living cultures and the extract, or autolysate, inoculated alternately. By the old method from 6 to 12 months were required to produce a meningococcic serum of high immunity value. By the new method a similar result has been achieved in from 8 to 12 weeks.

In following out this plan, a difficulty was early encountered, and one which was indeed foreseen. Two general methods of immunization of horses have been followed for the production of antimeningitis serum. In one the cultures of meningococci or the autolysate is injected subcutaneously (Flexner and Jobling[7]); in the other, the injections are made intravenously (Dopter[8]). In the first instance, no ill effects arise, aside from the occasional production of sterile abscesses. In the other, after several inoculations have been made, the horses become extremely sensitive, so that sudden death has been known to follow an injection of the culture or autolysate. This danger can, however, be prevented, as has been shown by Dopter,[9] by employing a desensitizing injection of the culture before making the full inoculation.

Hence the method adopted for the rapid production of the antimeningitis serum follows closely, but does not exactly reproduce, that already described for the rapid production of the antidysenteric serum. It consists in beginning with small doses of living meningococci injected daily for 3 days, followed by a period of rest of 7 days, when another series of injections is made. Thus for a 1,300 pound horse, one-twentieth of a 24 hour culture on slanted plain agar is injected on the 1st day. The material for injection is made up as follows: 2 cc. of physiological salt solution are added to a 24 hour agar slant

[7] Flexner, S., and Jobling, J. W., *Jour. Exper. Med.*, 1908, x, 141.

[8] Dopter, C., *Ann. de l'Inst. Pasteur*, 1910, xxiv, 96.

[9] Briot and Dopter, *Compt. rend. Soc. de biol.*, 1910, lxix, 174.

of meningococcus culture and the growth is suspended in it. Then 0.1 cc. of the suspension is transferred to 15 cc. of physiological salt solution and injected intravenously very slowly. The temperatures are taken hourly, beginning with the 4th hour after the injection, and continued until the temperature has reached its highest and begun to decline. 24 hours later 0.2 cc. of the suspension, and on the 3rd day 0.3 cc., that is about one-seventh of the agar slant, is used. After the lapse of 7 days, the dose given on the first of the 3 days corresponds with that given at the end of the last series; namely, in the instance in question, 0.3 cc. The temperature is again taken, and
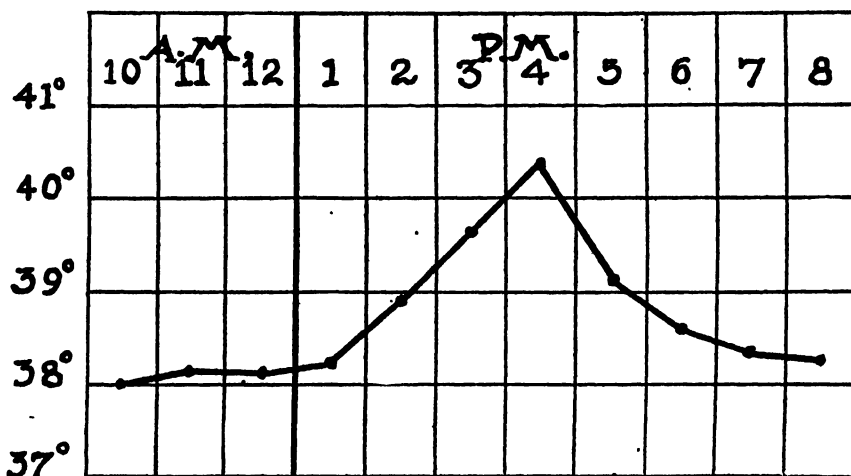
TEXT-FIG. 1. Typical febrile rise and fall following each single injection of meningococci.

if the rise does not equal 2.5–3°C. the conclusion drawn is that the dose has been too small. It is increased, therefore, for the injection 24 hours later above the usual rate of increase of 0.1 cc. according to the degree of rise of temperature. If the temperature does not fall to normal within 18 to 24 hours, the conclusion is drawn that the dose given has been too large (Text-fig. 1).

Following this plan, doses may be regulated with nicety and a maximum of reaction be obtained, we believe, with a minimum of danger. No serious effect is produced, although chills may attend the severer reactions. The greatest reaction, as a rule, is that pro-

duced by the first injection, whereas the succeeding injections on the 2nd and 3rd day tend to produce less severe reactions. Hence the increase between the second and the third injection may be larger than that between the first and second. In the second series of injections, the maximum doses have been from 0.4 to 0.45 cc., and in the third series from 0.5 to 0.6 cc. on the 3rd day. Since individual horses vary in their susceptibility, the first doses are small and the temperature curve forms the basis for adjusting the other doses (Text-fig. 2).

In Horse N only two series of injections were necessary before the typical and desired temperature curve was attained. The succeeding curves for any series agree closely with the third series in the chart.
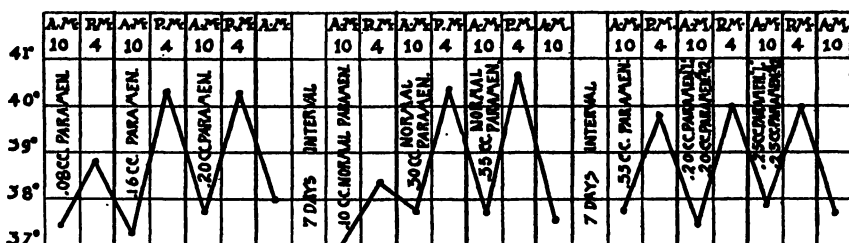
TEXT-FIG. 2.   Chart of Horse N showing febrile reactions and adjustment of doses in the first three series of injections.

In the succeeding series increase in the amounts injected is accomplished by the addition of new strains from time to time. For example, when the total dose is 0.6 cc. (*i.e.*, slightly more than one-third of the agar slant) it may consist of 0.2 cc. each of three strains. The largest amount of any single injection in a 1,300 pound horse has been one-fourth of each slant from seventeen different strains.

In preparing a polyvalent serum two slightly different procedures may be followed. According to one, the normal and the parameningococcus strains are inoculated within what may be designated as periods of two series; that is, the normal and the parameningococcus strains are alternated. According to the other, in which the autolysate is also employed, the period includes three series, one for the normal meningococcus, one for the parameningococcus strains, and one for the autolysate. The autolysate, in turn, is made up of equal parts

of a typical normal meningococcus and of a typical parameningococcus strain. Evidence is at hand, to be referred to below, which shows that the autolysate, at least in the sheep, excites little agglutinin formation while producing other protective principles.

Desensitization.

As stated, the horse becomes hypersensitive to the intravenous injections of the meningococci or its products apparently after the third or fourth series of injections. This reaction tends to be most severe after the first dose in each series, as might have been predicted. It was found that, acting upon the suggestion of Dopter,[9] desensitization may be effected apparently readily and certainly. Hence on the 1st day of each series about one-twentieth (later one-tenth) of a 24 hour slant of culture is injected intravenously, and 2 hours later the remainder of the dose is given. This desensitization suffices and need not be repeated on the 2nd and 3rd days, when the next doses are injected.

The danger from both brain and lung emboli must not be overlooked. Bull[10] has observed that suspensions of bacteria, introduced into the blood stream of an animal immune to the same organism, are clumped and deposited in the blood vessels of the brain, lungs, and in the spleen. If the bacteria are present in any considerable number, the clumps may occlude the cerebral and pulmonary capillaries and produce sudden death of the animal. We have found this to be true in the injection of meningococcus into normal and immune rabbits. It is possible that a part of the so called anaphylactic phenomena observed in immunization with meningococci can be explained by these facts, since the symptoms observed are similar if not identical in these two instances.

In order to avoid such a possible danger the suspensions of living meningococci are made up to a relatively larger volume, 15 to 20 cc., and introduced very slowly into the circulation.

[10] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 466.

Strains Employed in the Immunizing Process.

In the beginning a group of five representative normal meningococcus strains and a group consisting of an equal number of parameningococcus strains were used alternately in the series of injections. The progress of the formation of immune bodies in the serum was followed by studying the agglutination, opsonization, and complement deviation reactions, with as many strains of meningococci as possible. In this manner strains not employed in the original series of inoculations may be divided into temporary lots according to their reaction with the serum. If the reactions are not within or near the zone of reaction of the strains already being used for immunization, representatives of such lots are selected and placed in the immunizing group. The remaining members of these lots are again tested later when immune bodies against the representative of the group have appeared in the serum. Should the serum now contain antibodies effective against all members of the group, it will, of course, not be necessary to include them all. Agglutination serves as the most specific index of immunological reaction except in the case of inagglutinable strains, which, in our experience, are infrequently encountered.

By the above procedure we have studied sixty-four strains of meningococci, and from this number twenty-two were selected to be added to the two original immunizing groups which consisted respectively of five normal meningococcus strains and five parameningococcus strains. For convenience in regulating the doses and for injecting we have added equally to each group.

We selected for addition to the normal group eleven of those which showed the least variation from the normal meningococcus; and the remaining eleven, exhibiting wider variations, were placed in the second group which originally consisted only of parameningococci. The strains used at present for immunizing are thirty-two, and have for convenience been divided into two lots. Lot A consisted originally of five normal and irregular strains of meningococcus, and to these have been added eleven normal and irregular strains. Lot B consisted originally of five parameningococcus strains (two from the Pasteur Institute, Paris, and three isolated in America) and to these have been added eleven parameningococcus and irregular strains exhibiting wider variation from the normal. One lot is used for three

successive injections and after a rest of 7 days the other lot is used in like manner.   If care is not taken to balance the two lots, the immune bodies against one group may be developed to a greater extent than those against the other.   To avoid this, the serum is tested immunologically after every second series if living organisms are injected, and after every third series if autolysate is also given.   The relative doses of the two groups are regulated accordingly.

Since we have evidence that both normal meningococci and parameningococci differ among themselves in antigenic power, it is desirable to study all possible strains in the course of an epidemic in order that strains which are at variance with those being used in the production of an immune serum may be included in the immunizing groups.

This point is illustrated by the following experience with two strains.   Strain NO was tested against the polyvalent serum of a horse highly immune against at least twenty normal and parameningococcus strains and found to be agglutinated in a dilution of 1 : 10. This strain, which was later found to be parameningococcus, was included in the immunizing group, and after two inoculations the agglutinins rose to 1 : 100, and after four injections to 1 : 200.   When fewer strains are being used, the immune bodies may be developed more quickly.   Strain Andrews, brought from England by Dr. A. Gardner Robb, when tested against a potent serum effective against ten normal meningococcus and parameningococcus strains, agglutinated in a dilution of 1 : 10.   It was included in the immunizing group and after two series of injections, the agglutinating power of the serum for this strain rose to 1 : 200.   We consider that the larger doses of Andrews which it was possible to employ caused the more rapid rate in the production of the agglutinins.   Table I illustrates the development of agglutinins for strains relatively inagglutinable before their inclusion in the inoculation group.

TABLE I.

Strains.	Before injection.	After 1 injection.	After 2 injections.	After 4 injections.	After 5 injections.
Andrews........	1 : 10	1 : 100	1 : 200	....	1 : 2,000
NO............	1 : 10	....	1 : 100	1 : 200	....

Autolysate.

Equal parts of toluene autolysate[11] from normal meningococcus and from parameningococcus may be injected intravenously on 3 successive days forming one series, alternating with the two series of living meningococci. The serum of horses receiving the autolysate series develops agglutinins, opsonins, or power to deviate complement less quickly than that of animals receiving only the living organisms, though power to neutralize the toxin contained in the autolysate is developed.

TABLE II.

Serum.	Agglutinins.		Opsonins.		Complement deviation.	
	Normal meningo-coccus.	Paramenin-gococcus.	Normal meningo-coccus.	Paramenin-gococcus.	Antigen from normal meningo-coccus.	Antigen from paramenin-gococcus.
Sheep B (autolysate)............	1 : 100	1 : 50	1 : 50	1 : 1,000	1 : 200	1 : 100
Horse L (living cultures)........	1 : 5,000	1 : 2,000	1 : 2,000	1 : 5,000	1 : 10,000	1 : 5,000

A sheep received intravenously maximum doses of autolysate in series of 3 successive days and a rest of 7 days over a period of 9 months. Doses sufficient in size to cause marked febrile reaction and sometimes diarrhea, were administered. At the end of 9 months the serum exhibited only slight power of agglutination, opsonization, and complement deviation.

Table II shows the titer of the sheep serum compared with that of serum obtained from a horse receiving autolysate and also living meningococci.

The sheep serum when incubated with living meningococci and injected intraperitoneally into small guinea pigs was found to possess low anti-infectious value.

[11] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 105.

Immunity Value of the Antiserum.

The immune bodies of the horse serum were estimated by testing its agglutinating and opsonizing power with normal and parameningococcus strains and by determining its power to fix complement in the presence of antigens made from these strains.   Its anti-infectious power was determined by incubating varying amounts with one minimum lethal dose of living meningococci for 1 hour at 37°C., and injecting the mixture intraperitoneally into young guinea pigs weighing not less than 90 or more than 110 gm.

Agglutination.—Representative normal meningococci and parameningococci were selected for following the development of agglutinins.

TABLE III.

Horse L Serum.

Period.	Before injection.	1st.	2nd.	4th.	7th.*	After 6 wks. of autolysate injections.†	10th.	11th.‡	13th.	14th.
Meningococcus.......	1:30	1:60	1:80	1 : 1,000	1 : 5,000	1 : 2,000	1 : 2,000	1 : 500	1 : 1,000	1 : 2,000
Parameningococcus ..	1:10	....	1:80	1 : 200	1 : 2,000	1 : 500	1 : 1,000	1 : 1,000	1 : 1,000	1 : 2,000

* 7 periods extend over 10 weeks.   6 liters of blood were taken from the horse at this time.

† For 6 weeks after the 9th period the horse received autolysate only.

‡ Twelve new strains added.   1st period after bleeding.

Horse M Serum.

Period.	Before injection.	2nd.	4th.	7th.	10th.
Meningococcus..................	1 : 20	1 : 500	1 : 500	1 : 1,000	1 : 2,000
Parameningococcus.............	1 : 10	1 : 50*	1 : 1,000	1 : 1,000	1 : 2,000

10 periods extend over 12½ weeks.

* This low figure shows that the injections had not been properly balanced. Too few parameningococci had been injected; accordingly larger doses were given with the result that the agglutinins increased greatly during the next periods. The highest dilution at which any of our sera agglutinated was 1 : 5,000 for both normal and parameningococci.

The reactions were made at 55°C. and read after 24 hours. Table III shows the increase of agglutinins by periods.

Opsonins.—The opsonins were estimated by the Neufeld technique. Table IV shows their development by periods.

TABLE IV.

Horse L Serum.

Period.	5th.	7th.	9th.	Interval of 6 wks.	10th.	13th.	15th.
Meningococcus.	1 : 500	1 : 1,000	1 : 500*	Injected with auto-lysate only.	1 : 500	1 : 1,000	1 : 2,000
Parameningo-coccus.......	1 : 200	1 : 5,000	1 : 2,000		1 : 1,000	1 : 1,000	1 : 2,000

* Bled 6 liters just before this period.

Horse M Serum.

Period.	2nd.	7th.	11th.
Meningococcus..........................	1 : 500	1 : 200	1 : 2,000
Parameningococcus	1 : 200	1 : 1,000	1 : 2,000

Complement Fixation.—Tests for complement-binding bodies in the immune horse and sheep sera were made with antigens of regular normal meningococci as well as with those of irregular normal meningococcus and parameningococcus strains. The results are shown in Table V. It appears that in the serum from Horse L the power to bind complement ran fairly parallel with the power to agglutinate the meningococci.

TABLE V.

Complement Deviation by Serum L.

Period.	4th.	7th.	8th.	9th.	Interval of 6 wks.	10th.	11th.	12th.	13th.	14th.
Meningo-coccus	1:200	1 : 1,000	1 : 1,000	1 : 2,000	Injected with auto-lysate only.	1 : 1,000	1:500	1 : 1,000	1 : 1,000	1 : 1,000
Paramenin-gococcus ..	1:100	1 : 500	1 : 1,000	1 : 2,000		1 : 500	1:500	1 : 500	1 : 2,000	1 : 5,000

Complement Deviation by Serum M.

Period.	2nd.	4th.	5th.	6th.	9th.	12th.
Meningococcus	1 : 200	1 : 2,000	1 : 5,000	1 : 2,000	1 : 2,000	1 : 1,000
Parameningococcus....	1 : 200	1 : 1,000	1 : 2,000	1 : 1,000	1 : 1,000	1 : 5,000

Protective Value.—Flexner recommended the use of small guinea pigs in determining the anti-infectious power of antimeningitis serum. If young guinea pigs weighing between 90 and 110 gm. are used and the experiment is run in quadruplicate, some measure of the protective power is obtained. One minimum lethal dose of the living meningococcus is incubated with varying amounts of immune serum for 1 hour at 37°C. and then injected intraperitoneally.

Table VI shows the anti-infectious power of serum L compared with normal horse serum.

TABLE VI.

Anti-Infectious Power of Polyvalent Antimeningitis Serum.

Strain.	2 cc. of normal horse serum.	0.2 cc. of polyvalent immune serum.	0.3 cc. of polyvalent immune serum.	0.4 cc. of polyvalent immune serum.
1 m. l. d. of living meningococci.	No protection.	Protection in 1 out of 4.	Protection in 3 out of 4.	Protection in 4.
1 m. l. d. of living parameningo-cocci.	No protection.	Protection in 3 out of 4.	Protection in 3 out of 4.	Protection in 4.

If two minimum lethal doses are used, there is little protection after 48 hours. For example, in one series the control guinea pig receiving one minimum lethal dose and those receiving two minimum lethal. doses plus 0.5 cc. of serum died in 12 to 18 hours. Three out of four guinea pigs receiving two minimum lethal doses plus 0.6 cc. of serum lived between 47 and 48 hours.

Normal horse serum exerts practically no anti-infectious action either with meningococcus or with parameningococcus. Immune serum produced by the rapid method possesses a considerable degree

of anti-infectious power. About 0.4 cc. of this polyvalent serum is capable of neutralizing the infecting power of one minimum lethal dose of the living meningococcus or parameningococcus.

SUMMARY.

Potent antimeningitis serum can be safely produced in the horse by the method of three successive intravenous inoculations of living meningococci and parameningococci repeated at stated intervals.

Sudden and alarming symptoms and sudden death are avoided by employing first a desensitizing injection and then by adjusting the doses according to the febrile reaction and by making the highly diluted injections slowly.

Horses undergoing this process of immunization remain in good condition and may even gain in weight.

Specific immune bodies appear in the serum early and rise rapidly.

By inoculating alternately several strains of living meningococci and parameningococci, and the autolyzed products of each, a polyvalent serum of high titer can be produced in 8 to 12 weeks instead of in the 10 months required by the subcutaneous method.

The serum produced by this rapid method has been employed therapeutically in America, England, France, and some other countries.

It is highly desirable to isolate meningococci from many sources and test the strains against the polyvalent serum. Strains which are not agglutinated in high dilution in such a serum should be included subsequently in the lot of strains used for immunization.

THE INFLUENCE OF TYPHOID BACILLI ON THE ANTIBODIES OF NORMAL AND IMMUNE RABBITS.

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The view is held that bacterial injections cause a decrease in the antibody content of the blood of man and the lower animals.[1] This belief is largely based on the work of Wright in connection with various forms of bacterial inoculation. Wright[2] contends that every process of immunization produces a sequence of negative and positive phases, and hence that repeated inoculation may give rise to a multiple of such sequences. This phenomenon is of particular interest in connection with prophylactic typhoid vaccination and in the treatment of cases of typhoid fever with intravenous injection of typhoid bacilli or their products.

In the course of a study of native and acquired immunity of rabbits to typhoid bacilli, including the fate of the bacilli when injected intravenously,[3] certain observations were made which threw doubt on the action of the negative phase as expressed above. These incidental observations have now been extended and the complete results are reported in this paper.

Technique.

Immunization of Animals.—Rabbits were immunized to typhoid bacilli as follows: 1st day, 0.1 of an agar slant of heat-killed bacilli was injected into the peritoneum; 2nd day, 0.1 of an agar slant of heat-killed bacilli was injected into the vein; 5th day, 0.1 of an agar slant

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[1] von Wassermann, A., and Sommerfeld, P., *Med. Klin.*, 1915, xi, 1307.

[2] Wright, A. E., *Brit. Med. Jour.*, 1903, i, 1069.

[3] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 475.

of living bacilli was injected into the vein; 7th and 9th days, repetition of the 5th day treatment. 10 days after the last injection the sera gave agglutination reactions in dilutions varying from 1 : 30,000 to 1 : 200,000.

Antibodies.—The immune sera were tested for agglutinins, precipitins, opsonins, complement fixation, and bactericidins. Every reaction was not, however, carried out with the serum of each rabbit, but different groups of rabbits were often used in the different sets of experiments, as shown in the individual tables.

Agglutinins.—The fresh sera were diluted with 0.9 per cent sodium chloride solution, ranging in strength from 1 in 2 to 1 in 200,000, as the individual animals demanded, and one drop of a suspension of typhoid bacilli washed from a 24 hour agar slant (growth covering the entire surface of the slant) with 6 cc. of salt solution, was added to 1 cc. of each dilution. The quantity of bacilli was kept as uniform as possible in the various tests of the same animal's serum, since it is known that the titer of a serum depends largely upon the number of bacilli present.[4] The tubes were incubated at 37°C. for 2 hours and allowed to remain at room temperature for 2 hours before the final readings were made.

Complement Fixation.—Antigen was prepared as follows: Typhoid bacilli were grown on plain agar, washed from the agar with salt solution, and freed from particles of the medium by repeated centrifugation. The washed bacillary bodies were frozen and thawed several times, dried in vacuum over sulphuric acid, and ground to a fine powder in an agate mortar. 1 dg. of the powder was added to 100 cc. of salt solution, after which the mixture was shaken for several hours and then passed through a Berkefeld filter. The clear filtrate was used as antigen in both the complement fixation and precipitin tests. It proved highly satisfactory. From 0.05 to 0.1 cc. gave binding with 0.04 cc. of immune serum, while 0.3 cc. did not bind with normal serum. A hen-rabbit hemolytic system and guinea pig complement were employed.

Precipitins.—In testing for precipitins 0.2 cc. of the antigen was floated over 0.2 cc. of the undiluted sera and the results were read

[4] Foerster, O., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1897, xxiv, 500.

after standing for 2 hours at 37°C. The appearance of a delicate white ring at the plane of contact of serum and antigen was considered a positive reaction. The reaction was delicate and strictly specific. Filtrates from bouillon cultures of the bacteria gave nonspecific reactions.

Opsonins.—The opsonizing power of the sera was determined by the Neufeld method. Equal amounts of serum dilutions, bacterial suspension, and guinea pig leukocytes were mixed and incubated for 2 hours at 37°C.; slides were then made and stained after fixation in methyl alcohol with Manson's stain. The highest dilution of serum in which the degree of phagocytosis exceeded the salt solution control was considered as indicating the opsonic titer of the serum. This test was used more extensively in examining the normal than the immune sera. As typhoid bacilli are phagocyted in salt solution, the reactions are less delicate than the other tests employed.

Bactericidins.—The bactericidal capacity of the sera for typhoid bacilli was determined as described below.[5] Bacterial suspensions of varying strengths were prepared with sterile salt solution from a 24 hour bouillon culture. The suspensions ranged from 1 in 10 to 1 in 1,000,000. 0.05 cc. of the different suspensions was put in small testtubes, and 0.2 cc. of the serum to be tested was added to each tube. Care was taken to prevent the bacterial suspensions from touching the sides of the tubes above the level of the sera and thus to escape the action of the serum. The tubes were incubated for 1 hour, and 1 cc. of melted agar was added to each tube, and also to a set of salt solution controls. After incubation the degree of destruction can be easily determined. Sterility of the tubes or a reduction in the number of colonies to three or four was regarded as a positive reaction. A mere reduction of colonies is no doubt to be ascribed to agglutination. This test was used especially for the sera of normal rabbits and for rabbits after the first inoculation of bacilli.

[5] Wright, A. E., *Lancet*, 1901, i, 609.

Each type of experiment reported has been performed a number of times, but only a few tables illustrating the different classes will be given in detail.

In Experiment 1 (Table I) three rabbits having a high degree of immunity to typhoid bacilli were used. A sample of blood was taken from each rabbit, injections and bleedings were made, and the sera tested, as indicated in Protocols A, B, and C (Table I).

TABLE I.

Rabbit A.

Injection.	⅛ of an agar tube of typhoid bacilli.				
Time of bleeding.	Before injection.	1 hr.	5 hrs.	24 hrs.	48 hrs.
Agglutinins.....................	50,000	50,000	50,000	50,000	50,000
Precipitins.....................	+++	+++	+++	+++	+++
Complement fixation............	+++	+++	+++	+++	+++

Rabbit B.

Injection.	⅛ of an agar tube of typhoid bacilli.						
Time of bleeding.	Before injection.	1 hr.	5 hrs.	22 hrs.	47 hrs.	70 hrs.	120 hrs.
Agglutinins..............	80,000	80,000	80,000	80,000	80,000	80,000	100,000
Precipitins..............	+++	+++	+++	+++	+++	+++	+++
Complement fixation.....	+++	+++	+++	+++	+++	+++	+++

Rabbit C.

Injection.	⅛ of an agar tube of typhoid bacilli.				
Time of bleeding.	Before injection.	1 hr.	5 hrs.	24 hrs.	48 hrs.
Agglutinins.....................	30,000	30,000	30,000	30,000	30,000
Precipitins.....................	+++	+++	+++	+++	+++
Complement fixation............	+++	+++	+++	+++	+++

Experiment 1 shows definitely that an intravenous inoculation of typhoid bacilli causes no reduction in the concentration of agglutinins,

precipitins, and degree of complement fixation of the sera of rabbits up to time limits of the tests. This result was surprising. It was to be expected in view of Wright's results that an injection of the homologous bacteria would cause lowering of the various antibodies. No such reduction was detected.

The results of this experiment led to the performance of a large number of experiments of a similar nature, the number of bacilli inoculated being varied widely. It was found that the number of bacilli injected did not influence the end result. Injections were given under the skin and into the peritoneum with like results. Intravenous inoculation of the bacilli from an entire agar slant caused no reduction in the concentration of the antibodies. Lethal doses of bacilli were given and the rabbits bled while dying, but still the sera contained agglutinins, precipitins, and complement deviating antibodies in the same concentration present before the injections were made. Moreover, when sublethal doses were employed, the agglutinins were found to be increased at the 48th to the 96th hour after inoculation.

Natural and acquired antibodies show some points of difference: opsonins are thermolabile (Wright), bacteriotropins thermostabile (Neufeld); the natural agglutinins of rabbits for typhoid bacilli are thermolabile and disappear spontaneously within a few days, while the acquired agglutinins are thermostabile and persistent. Hence, it was thought possible that the bacillary injection might cause a reduction in the natural antibodies, while the acquired ones might remain uninfluenced.

Rabbits can be used advantageously for these experiments, since the sera of a large percentage contain natural opsonins, agglutinins, and bactericidal substances for typhoid bacilli. Representative protocols of experiments pertaining to the natural antibodies follow (Table II).

The protocols of Experiment 2 (Table II) represent three types of test. In one the number of bacilli injected was sufficient to cause acute intoxication and death in from 1 to 3 hours. Rabbits A[1] and B[1] fall in this group. From 1 to 2 hours after the injections the rabbits were in a state of collapse; they showed extreme relaxation, low blood pressure, heavy breathing, and, as a rule, diarrhea. Specimens of blood were taken while the rabbits were in this state or immediately

TABLE II.

Rabbit A[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915 Nov. 17	11.35 a.m. 11.40 a.m.	$\frac{1}{10}$ agar slant of typhoid bacilli in vein.	1–3+	1–15+	
	1.15 p.m.		1–3+	1–20+++	Rabbit moribund when bled and died immediately afterwards.

Rabbit B[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915 Nov. 17	11.25 a.m. 11.30 a.m.	Same as Rabbit A[1].	1–6+	1–15+	
	1 p.m.		1–6+	1–20+++	Blood taken from heart immediately after death.

Rabbit C[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915 Nov. 19 " 19	10 a.m. 12 m.	$\frac{1}{10}$ agar slant of typhoid bacilli in vein.	1–20+	1–40+	
" 19	2 p.m.		1–20+	1–40++	Heavy breathing; collapse; diarrhea.
" 19	5 p.m.		1–20+	1–40+++	Rabbit very weak.
" 20	10 a.m.		1–20+	1–90+	
" 21	9.30 a.m.		1–24++	1–90+	
" 22	8 a.m.		1–50+	1–300+	Rabbit found dead and unclotted blood removed from heart. Blood sterile.

TABLE II—*Continued*.

Rabbit D[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–12+	1–40+	
" 19	12 m.	Same as Rabbit C[1].			
" 19	2 p.m.		1–12+	1–40+	Collapse; diarrhea.
" 19	5 p.m.		1–12+++	1–60+	
" 20	9 a.m.		1–12+++	1–60+	
" 21	10 a.m.		1–16+++	1–80+	
" 21	11.45 a.m.	Rabbit died. Two agar slants and one tube of bouillon were each inoculated with one loop of heart's blood. One agar tube had two colonies, one was sterile; the bouillon gave a culture.			

Rabbit E[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–12+	1–30+	
" 19	10.20 a.m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
" 19	12.20 p.m.		1–12+	1–30+	Rabbit flaccid.
" 19	3.20 p.m.		1–12+	1–40+	
" 20	10 a.m.		1–12+	1–40+	
" 21	9 a.m.		1–12+	1–50+	
" 22	10 a.m.		1–24+	1–70+	
" 23	10 a.m.		1–160+	1–200+	
" 24	10 a.m.		1–160++	1–500+	
" 27	12 m.		1–300+	1–5,000+	
" 27	12.30 p.m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
" 27	2.30 p.m.		1–300+	1–5,000+	
" 27	6 p.m.		1–300+	1–5,000+	
" 28	9 a.m.		1–300+	1–5,000++	
" 29	9 a.m.		1–400+	1–20,000+	Rabbit in good condition.

TABLE II—*Concluded*.

Rabbit F[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	2 p.m.		1–12+++	1–40+	
	2.45 p.m.	$\frac{1}{10}$ agar slant of typhoid bacilli in vein.			
" 19	5 p.m.		1–16+	1–60+	
" 20	8.30 a.m.		1–12+	1–60+	
" 21	9 a.m.		1–20+	1–120+	
" 22	9 a.m.		1–40++	1–280+	
" 22	10.15 a.m.	$\frac{1}{10}$ agar slant of typhoid bacilli in vein.			
" 22	12.15 p.m.		1–40++	1–280+	
" 22	3.15 p.m.		1–40++	1–280+	
" 24	9 a.m.		1–60++++	1–400+	Rabbit in good condition

after death and compared for opsonins and agglutinins with the blood taken before the injection. The sera obtained subsequent to the administration of the bacilli agglutinated the bacilli more actively and in higher dilutions than those taken before the bacilli were injected. A few exceptions were noted, but a lowering of the agglutinating strength never occurred. An increase in opsonizing power was not observed; no difference could be detected between the sera in this respect before and after inoculation. This fact is probably due to the circumstance that the opsonic reaction is less delicate than the agglutination test.

In another series, represented by Rabbits C[1] and D[1], fewer bacilli were injected and the rabbits succumbed in from 1 to 3 days to a more chronic intoxication. The chief symptoms noted were emaciation, anemia, and loss of appetite. At autopsy, the blood was sterile or contained very few bacilli. The sera frequently showed an initial increase in agglutinating power a few hours after the inoculation, and then a steady increase in from 24 to 48 hours and continuing up to the time of death.

In the case of Rabbit C^1, the serum obtained before the bacilli were given showed agglutination in a dilution of 1 in 40, while at about the 72nd hour the reaction was present in a dilution of 1 : 300. This rabbit was markedly anemic; no increase in opsonizing power was detected until 24 or 48 hours after the inoculation, at which time an increase was evident.

A third group is represented by Rabbits E^1 and F^1. Here repeated sublethal inoculations were made. The first injections were followed by results similar to those in Rabbits C^1 and D^1; namely, an initial increase in agglutinins and a gradual rise in opsonins and agglutinins from the 24th to the 48th hour on. The second injections were followed by a marked increase in both opsonins and agglutinins, beginning at the 24th to the 48th hour without any intervening negative phase.

A separate group of rabbits was used for the bactericidal tests, as it was often difficult to obtain enough blood from one rabbit at the various bleedings for all the tests. Protocols representing these experiments are tabulated in Table III.

The results of Experiment 3 (Table III) show a marked increase in the bactericidal power of the sera obtained from the blood taken at short and frequent intervals after the bacilli were inoculated. Five- or tenfold increase was noted 3 hours after the injection, and the increase was still greater at the expiration of 24 hours. The control, Rabbit C^2, was used to determine whether the variations observed with the sera from the inoculated rabbits were due to the different ages of the sera at the time of the tests, for they were from 1 to 24 hours old when tested. The results obtained suggest rather that remaining on the clot over night increases the bactericidal power of the sera. At least it may be said that the age of the sera was not the cause of the effects obtained. It is also evident that the sera of different rabbits vary considerably in bactericidal as they do in opsonic and agglutinating capacity. The increase in bactericidal power is not so pronounced when fewer bacilli are inoculated; $\frac{1}{10}$ or $\frac{1}{40}$ of an agar slant caused only a slight increase. It appears that a severe intoxication is necessary to cause this mobilization of the bactericidal substances.

TABLE III.

Rabbit A².

Injection.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
1/10 agar slant in vein.	Before injection.	0.2	100
	3 hrs. after "	0.2	10,000
	5 " " "	0.2	10,000
	24 " " "	0.2	1,000,000

Rabbit B².

Injection.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
1/10 agar slant in vein.	Before injection.	0.2	10,000
	3 hrs. after "	0.2	50,000
	5 " " "	0.2	1,000,000
	This rabbit died 5¼ hrs. after injection.		

Rabbit C².

Injections.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
No injection.	Blood taken when the other rabbits were bled and tested at the same time.		
	Before injection.	0.2	1,000,000
	3 hrs. after "	0.2	1,000,000
	5 " " "	0.2	100,000
	24 " " "	0.2	100,000

DISCUSSION.

The data presented in this paper show conclusively that inoculation of typhoid bacilli causes no reduction in the concentrations of the natural or acquired antibodies present within the blood. Notwithstanding the fact that a decrease was expected, it was found that neither subcutaneous nor intraperitoneal nor intravenous injections sufficed to bring about the negative phase of Wright. It might have been supposed that large intravenous injections would remove the anti-

bodies from the blood as large quantities of cultures do from the serum *in vitro;* but no such effect was detected. This result may receive partial explanation from the fact that bacteria are quickly removed from the circulation through agglutination and accumulation in the organs and tissues,[6] but obviously the quantity of agglutinin thus engaged affects but little the whole quantity present.

Attention is especially drawn to the fact not only of the absence of the negative phase, but to the actual increase of the natural antibodies following the intravenous inoculations of the bacilli. The time required—from 1 to 3 hours—for the increase to become manifest is obviously too short for actual production to occur; the only alternative seems, therefore, a mobilization of preformed antibodies from the internal organs. Just what the source of the mobilized antibodies is has not been determined. The inoculations cause extensive destruction of leukocytes; but whether their disintegration liberates the antibodies cannot be stated. It is convenient to imagine that the beneficial effects said to follow the treatment of typhoid fever by means of the intravenous injection of specially prepared typhoid bacilli may be ascribed to this rapid mobilization of antibodies.

SUMMARY.

The subcutaneous, intraperitoneal, or intravenous inoculation of cultures of typhoid bacilli did not cause, as far as could be determined, a decrease in the antibody content of the blood serum of the rabbit.

On the other hand, the intravenous inoculation of typhoid bacilli causes a rapid mobilization of normal antibodies, thus increasing their concentration in the blood, to be followed somewhat later, as in the other forms of inoculation, by the production of so called acquired antibodies.

No such condition as the negative phase of Wright was discovered, although especially looked for in the experiments.

I am indebted to Miss Ida W. Pritchett for technical assistance in connection with the above study.

[6] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 475.

KLOSSIELLA INFECTION OF THE GUINEA PIG.

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PLATES 63 TO 70.

In studying the lesions produced by arsenical compounds in the kidneys of different species of animals, certain parasites were observed in the renal tubules of guinea pigs, which strikingly resembled the coccidium, *Klossiella muris*, observed and described by Smith and Johnson[1,2] in the kidneys of the mouse. As we believed the guinea pig to be relatively free from spontaneous renal lesions, a search through the literature was made to ascertain if renal parasites of the guinea pig had been previously described. At the same time the study of a large number of guinea pig kidneys was undertaken, in order to determine the relative frequency of this parasite as well as to obtain information concerning the lesions which tend to be associated with it.

Seidelin[3] in 1914 describes a parasite which occurred in the kidneys of two guinea pigs from Nigeria, and which bears such strong resemblance to *Klossiella muris* in some of its stages that he considers that both parasites "must be regarded as belonging to one and the same genus, whilst the question of their specific identity or otherwise cannot at the present time be finally decided." He further states that the parasite does not appear to be a common one in West African guinea pigs, as he found only one case among about twenty guinea pigs of the Yaba series. Later he found identical parasites in sections of a kidney of another guinea pig from the same locality. He found no similar instance in guinea pigs procured in Liverpool.

[1] Smith, T., *Jour. Comp. Med. and Surg.*, 1889, x, 211.

[2] Smith, T., and Johnson, H. P., *Jour. Exper. Med.*, 1914, vi, 303.

[3] Seidelin, H., *Ann. Trop. Med. and Parasit.*, 1914–15, viii, 553.

Seidelin describes some of the stages of the parasite detected by him and suggests a probable life cycle. There is undoubtedly a close resemblance between this parasite and *Klossiella muris*, and again between both these parasites and the one about to be described. On the other hand, the question of the identity of Seidelin's parasite and the one we have observed will be discussed later on in this paper. There are certainly points of similarity between the two, as well as some points of difference.

It may be stated here that we have found the infection to be by no means uncommon in kidneys of guinea pigs. In sixty guinea pigs examined for renal parasites, twelve were found to be infected. In other words, in our experiments with guinea pigs we found that we must reckon upon at least 20 per cent of the animals from our sources being infected with this renal parasite.

The material examined consisted of kidney sections of sixty adult guinea pigs from two sources, one in Philadelphia, the other in New Brunswick, New Jersey. These pigs were either perfectly normal animals or were animals which had been used for toxicological experiments with various arsenical compounds and had survived the use of the drug not more than 24 hours. This arbitrary time limit was set in the choice of material in order that any chronic pathological process observed might not be confused with the drug action, since within this time (24 hours) no extensive proliferative change in the kidney can be attributed to the action of these compounds. The kidney tissue was fixed in Zenker's fluid and imbedded in paraffin. Sections were stained with hematoxylin and eosin, methylene blue and eosin, and Giemsa's stain. In addition, frozen sections were made from tissue fixed in 10 per cent formalin and stained with hematoxylin and eosin.

Description of the Parasite.

Various stages of the parasite have been seen in the kidneys of guinea pigs and in order to facilitate an interpretation of its probable life cycle, a certain sequence of description will be observed, beginning with the stage or form most frequently encountered.

Sporoblast Cycle.—The form most frequently seen is a small round or ovoid body measuring 7 to 8 μ in diameter. It stains a very light

bluish pink with hematoxylin and eosin, and contains numerous small dark blue masses of chromatin scattered through the cytoplasm in no definite arrangement (Figs. 1 and 2). The cytoplasm is somewhat granular and slightly, if at all refractile. The chromatin is of two sizes, larger irregular masses and tiny pin points, both taking a dark blue stain with hematoxylin and methylene blue. Usually several of these parasites are found free in the lumen of the renal tubules and sometimes the lumen appears to be completely blocked with them. They are most frequently seen in the straight tubules of the inner half of the cortex, but are also found somewhat less numerously in the convoluted tubules and in the tubules of the upper medulla. Occasionally one or two may be found in the capsular space of the glomerulus. This stage of the parasite is apparently very similar to the so called "daughter sporoblast" stage of *Klossiella muris* described by Smith and Johnson.

In certain sections it has been possible to make out a confining cell membrane surrounding several of these bodies, as in Fig. 2. The membrane is all that remains of the epithelial cell that originally contained the developing parasites. As they grow and increase in size the host cell must necessarily enlarge until it becomes flask-shaped and bulges out into the lumen of the tubule. The parasites occupy the broad distal portion of the containing cell and the nucleus is usually crowded inwards and often to one corner of the cell. It frequently appears flattened, compressed, or shrunken, and eventually it disappears, apparently just preceding or about the time of rupture of the cell. The cytoplasm of the host cell gradually becomes more and more scanty and granular and finally all that remains of the cell is its enormously distended cell wall, which is ruptured by the escaping parasites. It is somewhat difficult to determine just how many parasites develop in the epithelial cell, as we have had no opportunity of examining any material except cross sections of kidney tissue. However, there are probably at least ten to twelve.

Each of these parasites next undergoes a division or segmentation into eight to twelve bodies (Fig. 3). These bodies are falciform or banana-shaped and are extremely small, measuring 1 by 4 to 5 μ, and are surrounded by a distinct membrane. Their cytoplasm is clear and non-granular, pink (hematoxylin and eosin, and Giemsa's

stain), and each body contains a dark blue dot of chromatin. We believe that these tiny fusiform bodies are similar to the form designated as the sporozoites of *Klossiella muris*. The further history of these bodies is entirely conjectural and will be referred to later in the discussion.

Not uncommonly one will find in heavily infected kidneys a form (Fig. 4) which is evidently the precursor of the two stages just described. This form is comparatively large, and may measure 17 by 22 μ; it has the same appearance and staining characteristics as the smaller more usual forms first described (Figs. 1 and 2). A breaking up of this large mass into several constituent smaller bodies results in a picture similar to Fig. 2. In studying various infected kidneys, we have seen several microscopic fields in which there were all gradations of this division (Fig. 6), so that we are inclined to regard this large parasitic mass as corresponding to the so called "mother sporoblast" stage of *Klossiella muris*.

Ring Form.—In several of the sections of heavily infected kidneys we have seen a stage of the parasite which we have called the "ring," or "annular" form (Figs. 7 and 8). It is always within an epithelial cell and the cell itself is enlarged and may protrude into the lumen of the tubule to the extent of nearly occluding it, as in Fig. 8. This form measures 18 to 20 μ in diameter and consists of a series of twelve to eighteen definite divisions or segments arranged in a circle or ring. Each division is extremely small, ovoid in shape, and measures 3 by 5 to 7 μ. They are smaller than the more frequently encountered forms seen in Figs. 1, 2, 3, and 4 (the daughter sporoblasts), and in appearance are totally different. The cytoplasm is refractile and stains practically not at all, or at most a very pale pink with hematoxylin and eosin, and Giemsa's stain. Each segment contains one or two chromatin dots. In some instances the two chromatin dots appear to be fusing together; in others they are distinctly separate from one another. In other instances the one chromatin dot is elliptical in shape, and it is possible that the finding of two chromatin dots is due to the level of the section. In the center of the ring form is a small amount of a granular refractile pale pink staining material which is apparently a residuum of the cytoplasm of the surrounding ring segments (Figs. 7 and 8).

In Fig. 8 there is a typical ring body and another intracellular form which we think is also a ring body seen from the outside. In other words, a ring body is a section through such a hollow sphere as is seen in Figs. 9 and 10. Here there are eighteen small segments or divisions, each of which is similar in appearance and staining reactions to the segments of the ring form.

The ring segments are not only considerably larger than the final divisions of the sporoblast cycle, the sporozoites (Fig. 5), but are also of a totally different shape and appearance. Moreover, the immediate precursor stage, the ring form, is still enclosed in an epithelial cell, while the precursor stage of the falciform bodies shown in Fig. 5 is not necessarily intracellular. Indeed, when the division into the tiny falciform bodies or sporozoites occurs, the daughter sporoblasts are probably always extracellular and free in the lumen of the tubule. In addition, the sporozoites are contained in a very distinct spore, having a definite membrane, while there is no such structure surrounding the segments of the ring form. When the ring divides, the resulting segments are all apparently set free in the remains of the epithelial host cell and eventually in the lumen of the tubule when this cell ruptures. The ring segments seem to be identical with still another stage of the parasite about to be described and may represent its earliest and youngest form.

Hyaline Forms.—Figs. 11 to 16 illustrate a single stage of the parasite which from its appearance and staining character is evidently one of the segments of the ring form. It is very small, measuring from 5 to 8 μ in length and 3 to 5 μ in breadth, and is oval or ovoid in shape. The cytoplasm is non-granular, extremely refractile, hyaline, and stains pink with hematoxylin and eosin, or Giemsa's stain. It contains a relatively large clear-cut mass of dark blue staining chromatin placed towards one end of the parasite. At the opposite pole a small clear non-staining area may be seen, as in Fig. 12. In the section from which Fig. 11 was taken this clear area is visible, but it is very small and barely shows in the photograph. This stage measures 3 by 5.5 to 7 μ. It may be extra- or intracellular, as shown in the illustrations. In Fig. 12 it apparently is about to enter an epithelial cell of a renal tubule. In Fig. 11 it is clearly intracellular and surrounded by a vacuole. The nucleus of the host

cell is immediately below the parasite.   The epithelial cell opposite
the parasite on the other side of the renal tubule contains an ovoid
inclusion which is probably a similar parasitic body, although the
mass of chromatin is not seen in the section.   The cytoplasm of the
two cellular inclusions is identical.

In Fig. 13 another intracellular parasite is seen and here the chroma-
tin has divided into two distinct parts.   The parasite itself measures
5 by 7 μ.   The host cell has become enormously enlarged and pro-
trudes into the lumen of the tubule.   Fig. 14 shows a further division
of the chromatin into four distinct segments.   This parasite is also
intracellular, but in order to bring the chromatin into focus for the
photograph, the epithelial cell outlines are not seen.   Just below
this parasite is another smaller intracellular form similar to those
shown in Figs. 11 and 12, with only one chromatin mass.

Schizogonic Cycle.—Fig. 17 illustrates a spherical form which we
are inclined to believe represents the schizogony of the parasite.   It
it comparatively large—measuring 22 μ in diameter, and is composed
of a large number (thirty to forty) of tiny fusiform bodies or mero-
zoites enclosed in a rather indistinct and apparently very thin mem-
brane.   It does not appear to be enclosed in an epithelial cell, but
is extracellular and free in the lumen of the renal tubule.   The mero-
zoites in longitudinal section measure 1.5 by 7 to 8 μ.   Their cyto-
plasm stains pink with hematoxylin and eosin, and each contains a
tiny dot of chromatin.   These small falciform bodies are extremely
similar to the sporozoites described above.   The general appearance
of this large form suggests its similarity, if not identity with the
glomerular body of *Klossiella muris*, which Smith and Johnson in-
terpret as the schizogony of the mouse parasite.   We have not found
this form in the capsular space of the glomeruli but in the convoluted
tubules of the first order.   It is not a common stage and we have
found it in only one kidney.

*Pathological Changes in the Kidneys of Guinea Pigs Associated with
the Parasitic Infection.*

The kidneys of guinea pigs infected with the parasite show cer-
tain pathological changes of a chronic nature which we are inclined
to attribute to the presence of the organism.   They have been found

in all cases in which the parasite has been seen, and in several instances where these lesions were observed the infection was very light, and the parasite was found only after a thorough search.

The lesions which we think are caused by the parasite consist in an irregular accumulation of fibroblasts and small round cells about the base of some of the glomeruli. Both the distribution and arrangement of this infiltration are very irregular (Figs. 18 to 21). In a single microscopic field one or two glomeruli may be affected in this manner, while the adjacent glomeruli are normal in appearance. Moreover, the extent of the infiltration varies considerably. Some glomeruli have only a slight accumulation of round cells about their base, others are almost obliterated, as in Figs. 18, 20, and 21. Usually there seem to be relatively more cells of the small round mononuclear type than fibroblasts. The fibroblasts themselves are apparently not young cells.

These cellular accumulations seem to be fairly well confined to the immediate vicinity of the glomeruli. In certain instances, however, the round cells and fibroblasts extend outward to a limited degree into the labyrinthine tissue and along the medullary rays between the tubules, but in these cases the connection between this extension and the accumulation of similar cells about the neighboring glomeruli can be easily traced.

There seems to be no reaction of the kidney tissue in the immediate vicinity of the parasite itself, that is, in the inner half of the cortex where we have found the parasite to be most numerous. Here one may see half a dozen consecutive tubules filled with parasites and eight or ten epithelial cells containing ring forms, yet there is apparently no abnormality in the immediate interstitial connective tissue. The glomeruli, however, just above these infected tubules show a more or less extensive infiltration of round cells and fibroblasts. The portion of medulla just below the infected tubule shows no appreciable change. No gross changes in the kidneys referable to the renal parasite have been noted.

<center>DISCUSSION.</center>

The description of the various stages of the parasite found in the kidney of the guinea pig has been arranged so as to relate, tentatively at least, those forms which seem to belong to the same cycles

of development.   Certainly two different cycles of development have
been observed.   The exact interpretation of the cycles must be more
or less hypothetical, since there is an obvious lack of knowledge of
all the stages in the evolution of the parasite.

The first cycle, described under the sporozoites is similar to the
so called sporoblast stage of *Klossiella muris* and is evidently similar
to the stage described by Seidelin.   However, we have never seen
more than twelve daughter sporoblasts, and usually only eight to
ten resulting from the first division of the mother or pan sporoblast
form, while Seidelin gives sixteen to twenty as the probable number.
This discrepancy, if the two parasites are identical, can probably
only be settled by the study of fresh material or an extensive
series of sections.   In the second division, into sporozoites, we have
never observed more than twelve, each sporozoite measuring 1 by
4 to 5 μ.   Seidelin describes thirty sporozoites, each measuring 1.5
by 8 μ.   If this difference in the number of sporozoites continues
to hold after the study of fresh tissue, we shall be inclined to believe
that the two parasites belong to different species.

That this cycle of the parasitic development represents the sporo-
blast phase is strongly suggested by the fact that the sporozoites
are confined in what is apparently a typical spore.   We have never
seen these spore-like bodies breaking up in the lumen of the kidney
tubules, and it is probable that the spores containing the sporozoites
are excreted in the urine, which is afterwards swallowed by the same
or other guinea pigs, and that the spore membrane is digested away
by the gastric juice, thus freeing the sporozoites.   The extremely
small size and fusiform shape of these tiny bodies would doubtless
enable them to pass through the gastric or intestinal mucosa into the
blood stream and so into the kidney.   On the other hand, we have
never seen any sporozoites in the glomerular tuft or capsular space.

The ring forms are not numerous, but they are easily found in
heavily infected kidneys.   They differ markedly from any stages of
the sporoblast cycle and do not appear to be an integral part of it.
It is easy to differentiate between a mother sporoblast, for instance,
and a ring form seen from the outside, or cut on a tangent, so that the
annular appearance is not seen.   Moreover, the resulting division or
segments of the two forms are apparently very dissimilar.   The

sporozoites are slender fusiform bodies, 1 by 4 μ, with a tiny dot of chromatin; the ring segments are oval or ovoid, 3 by 5 to 7 μ, with a relatively large mass of chromatin. The ring segments are apparently identical with the small hyaline forms seen free in the lumen of the renal tubules or in the epithelial cells, and there is nothing in the nature of a spore membrane about the dividing ring to prevent their escape into the tubules when the ring completely segments. The final interpretation is, of course, one of conjecture only, but the cycle is strongly suggestive of a sexual phase. The comparatively large number of ring segments or hyaline forms suggests further that they may be the microgametes, and that the ring form may be the microgametocyte. We have not seen any bodies which we could interpret as macrogametes, or any process of fertilization or conjugation, unless Fig. 16 represents this phase.

Smith and Johnson describe in the sporoblast stage of *Klossiella muris* a budding process in which the chromatin occupies the periphery of the budding masses. One might think that the ring form is simply a cross section of some of these buds of the mother sporoblast. But the ring form possesses not only a different type of protoplasm from the mother or daughter sporoblast, but in addition its segments are totally unlike either the daughter sporoblasts on the one hand or the sporozoites on the other.

The asexual or schizogonic cycle is apparently represented by the large extracellular segmenting form seen in Fig. 17. It resembles the glomerular body described by Smith and Johnson and interpreted by them as the schizogonic form. We have not seen it in the glomerular space, but only in the convoluted tubules and its scarcity may be tentatively explained on the assumption that such a stage is present only in early and light infections. Later, apparently, the sporoblast cycle may supersede the schizogony, for this cycle only has been detected in our specimens of heavily infected kidneys. Its extremely large size and the great number of its segments or divisions preclude its belonging to either the sporoblast or ring cycles. Moreover, its situation free in the convoluted tubules is one of the locations where one would expect to find the development of such a stage, if, as we have already suggested, the infecting sporozoites find their way into the kidney by the blood stream. The merozoites or

segments of this large body are similar to the sporozoites, the final divisions of the sporoblast cycle. The conspicuous difference between the two stages of segmentation, aside from the difference in their number, is that the sporozoites are enclosed in a definite spore membrane, while the existence of a membrane surrounding the merozoite is problematical. In the specimens we have seen there is a very indistinct membrane, which, as in Fig. 17, is apparently ruptured, allowing the escape of the merozoites into the renal tubules.

Seidelin found no glomerular bodies as described by Smith and Johnson for *Klossiella muris*, but he considers that some of his tubular forms appear identical with the glomerular forms depicted by them. Unfortunately, Seidelin gives no illustration of this particular tubular form, so that we are unable to compare satisfactorily the two apparently corresponding stages. Seidelin is inclined to the opinion that these tubular forms represent the schizogony. Smith and Johnson think that their glomerular body is the schizogony for *Klosiella muris* and that the tubular forms are stages in the sporoblast cycle.

The pathological changes in the kidney of guinea pigs which we associate with the presence of this parasite are slight but definite and consist of an irregular accumulation of round cells and fibroblasts about some of the glomeruli. There is but slight involvement of the labyrinthine tissue adjacent to the glomeruli and apparently none at all in the lower or inner half of the cortex where the majority of the parasites are found. This may be due to the fact that the infecting sporozoites enter the kidney by way of the glomerular capillaries and that here the most serious injury to the kidney occurs, with a subsequent infiltration of round cells and fibroblasts.

<div align="center">SUMMARY.</div>

We have found in the kidneys of twelve supposedly normal guinea pigs, coming from Pennsylvania and New Jersey, a parasite that closely resembles in some of its phases *Klossiella muris*, described by Smith and Johnson, and the renal parasite of two West African guinea pigs, described by Seidelin.

The forms most commonly found by us and described as the sporoblast cycle, are evidently similar to those described by Smith

and Johnson and by Seidelin. There are certain discrepancies of measurement between the parasite described by Seidelin and the one here described, but the most important difference between the two is the different number of sporozoites resulting from a final division of the daughter sporoblasts. Seidelin has found thirty sporozoites; we have found from eight to twelve, while the usual number is eight. Further, we have found a ring form which is unlike any of the stages in either the sporoblast or schizogonic cycle, and which we interpret tentatively as the male element or microgamete. In addition, we have found a tubular form which resembles the glomerular body of *Klossiella muris* and which we think is the schizogonic phase of this parasite.

EXPLANATION OF PLATES.

The illustrations are all from untouched microphotographs except Fig. 8, which is a drawing of an actual microscopic field. All the specimens except Fig. 17 are from Zenker fixed tissue.

PLATE 63.

FIG. 1. Renal tubules showing a heavy parasitic infection in different stages, the majority being daughter sporoblasts. Hematoxylin and eosin. × 675.

FIG. 2. Eight daughter sporoblasts enclosed in an epithelial cell of a convoluted tubule. Hematoxylin and eosin. × 1,000.

FIG. 3. Three spores containing seven to nine sporozoites. Note the distinct spore membrane. Hematoxylin and eosin. × 1,000.

PLATE 64.

FIG. 4. Mother sporoblast enclosed in an epithelial cell. The cell outline is not distinct in the photograph. Giemsa's stain. × 1,000.

FIG. 5. Glomerulus containing a single parasite, probably a young mother sporoblast in the capsular space. Hematoxylin and eosin. × 1,000.

FIG. 6. Three greatly enlarged epithelial cells, two containing mother sporoblasts, the third in the center containing eleven daughter sporoblasts. Hematoxylin and eosin. × 1,000.

PLATE 65.

FIG. 7. Two ring forms in adjoining tubules, each enclosed in an enlarged epithelial cell. The ring to the right shows ten divisions. Hematoxylin and eosin. × 1,000.

FIG. 8. Two ring forms in distended epithelial cells; the lower form is seen from the outside. Hematoxylin and eosin. × 1,000.

PLATE 66.

FIG. 9. Two ring bodies and several daughter sporoblasts. The ring body on the left is a tangential section through the edge of a hollow sphere. Methylene blue and eosin. × 1,000.

FIG. 10. The same field as in Fig. 9, but at a lower level, showing that the so called ring body is a section through a hollow sphere. Methylene blue and eosin. × 1,000.

PLATE 67.

FIG. 11. Two intracellular hyaline forms, the one to the right showing chromatin. Hematoxylin and eosin. × 1,000.

FIG. 12. An extracellular spindle-shaped hyaline body showing the chromatin mass at one pole and a clear non-staining area at the opposite pole. One extremity of this hyaline form is in the protoplasm of an epithelial cell. Hematoxylin and eosin. × 1,000.

FIG. 13. An intracellular hyaline form in an enlarged epithelial cell. The chromatin has divided into two masses. The parasite is in a vacuole in the host cell. Hematoxylin and eosin. × 1,000.

FIG. 14. Two intracellular hyaline forms; the chromatin of the upper parasite has divided into four masses. Giemsa's stain. × 1,000.

PLATE 68.

FIG. 15. Two small intracellular hyaline forms. Giemsa's stain. × 1,000.

FIG. 16. Two intracellular hyaline bodies. The larger one shows two elongated chromatin masses. The smaller parasite to the left is at a level which shows no chromatin. Giemsa's stain. × 1,000.

FIG. 17. A schizogonic form showing thirty to forty merozoites. This body is free in the tubule and has no definite membrane surrounding it. Taken from a frozen section fixed in 10 per cent formalin. Hematoxylin and eosin. × 1,000.

PLATE 69.

FIG. 18. Irregular cellular infiltrations, especially about the base of some of the glomeruli. Hematoxylin and eosin. × 125.

FIG. 19. Three glomeruli showing very slight cellular infiltration about the base. Two glomeruli show no such infiltration. Hematoxylin and eosin. × 210.

PLATE 70.

FIG. 20. The two lower glomeruli show a slight irregular accumulation of round cells and fibroblasts. The upper glomeruli are normal. Hematoxylin and eosin. × 210.

FIG. 21. The two glomeruli to the right show a fairly extensive accumulation of round cells and fibroblasts with an irregular extension into the labyrinth. The glomeruli to the left are practically normal. Hematoxylin and eosin. × 210.
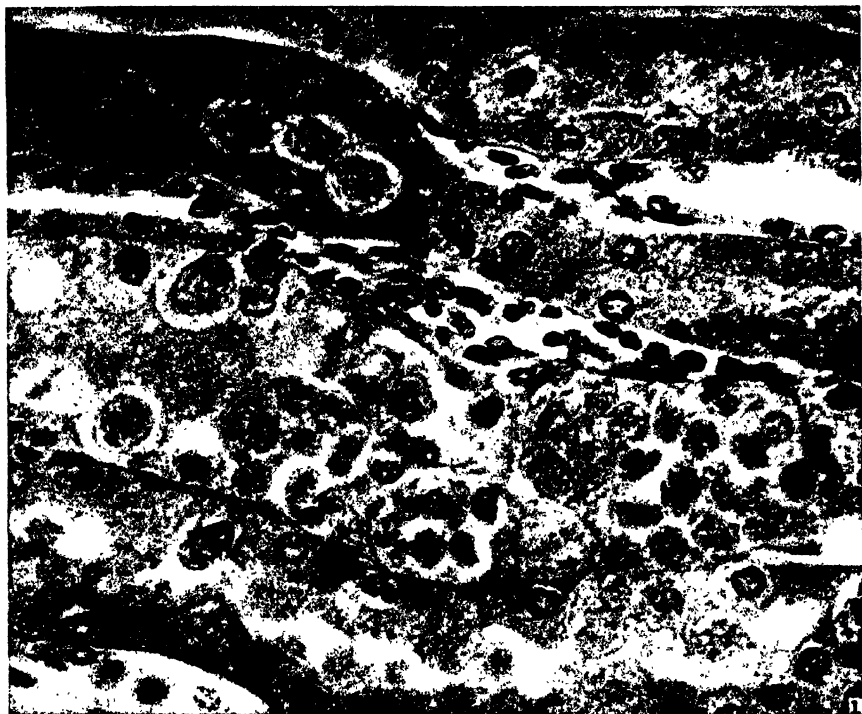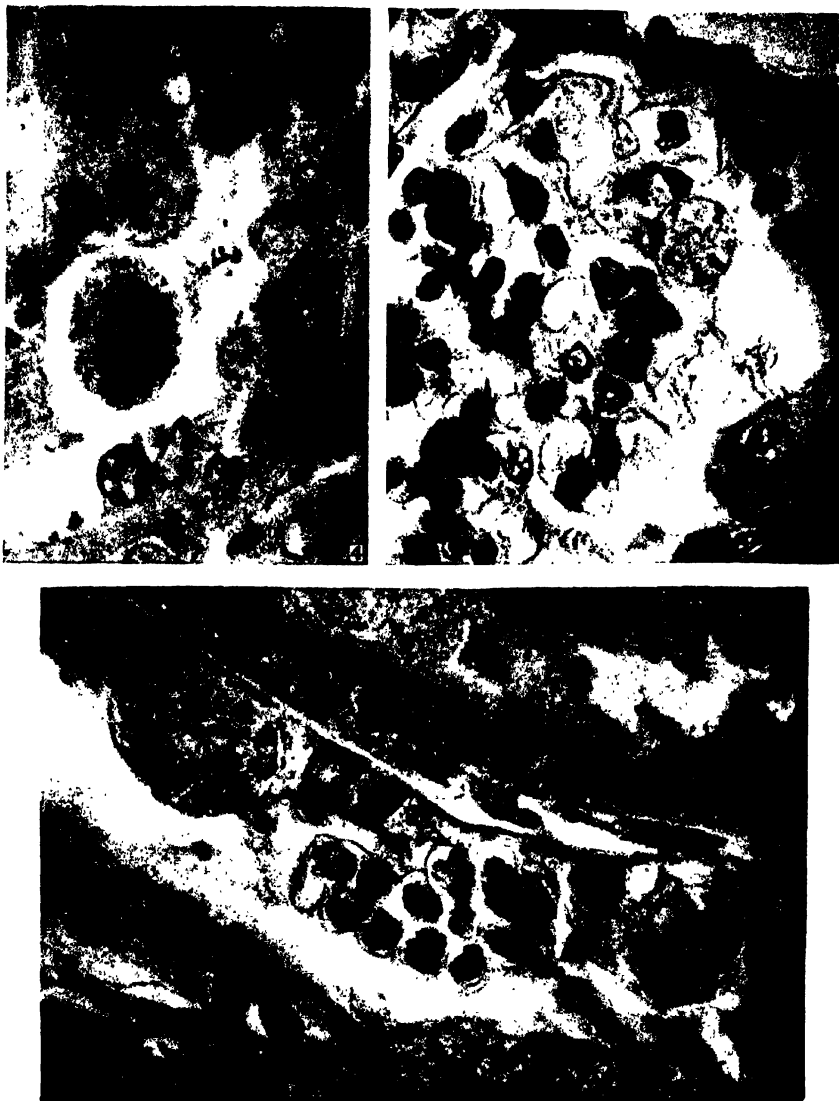
PLATE 64.
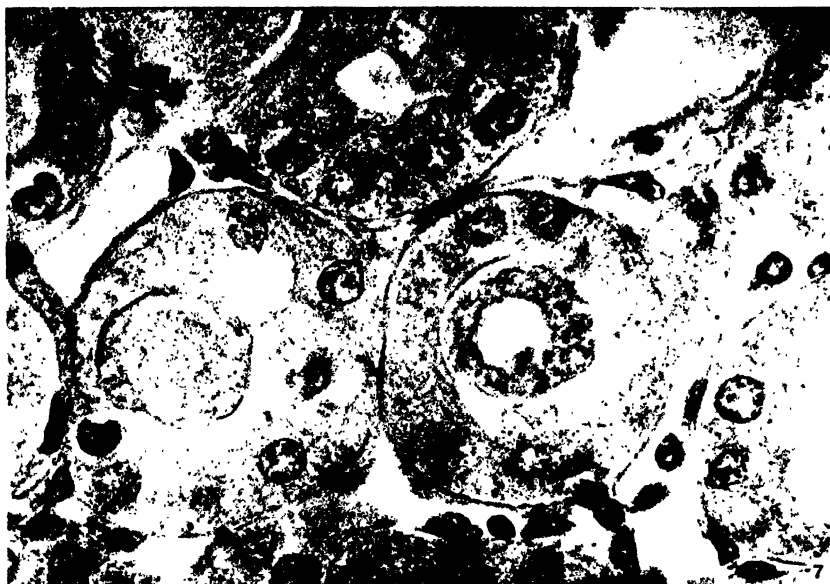
(Pearce: Klosiella Infection of the Guinea Pig.)

(Pearce: Klosiella Infection of the Guinea Pig.)
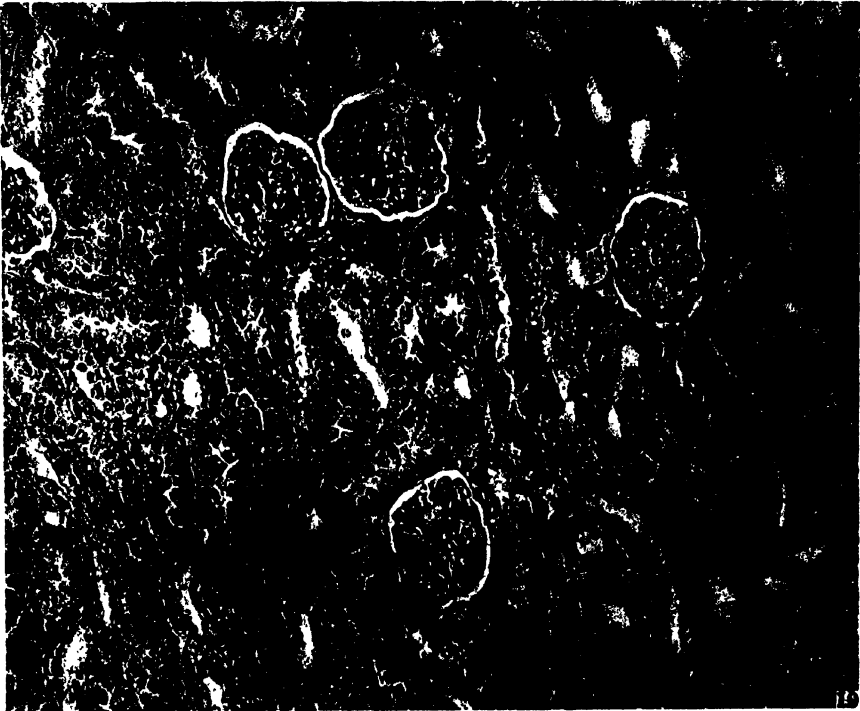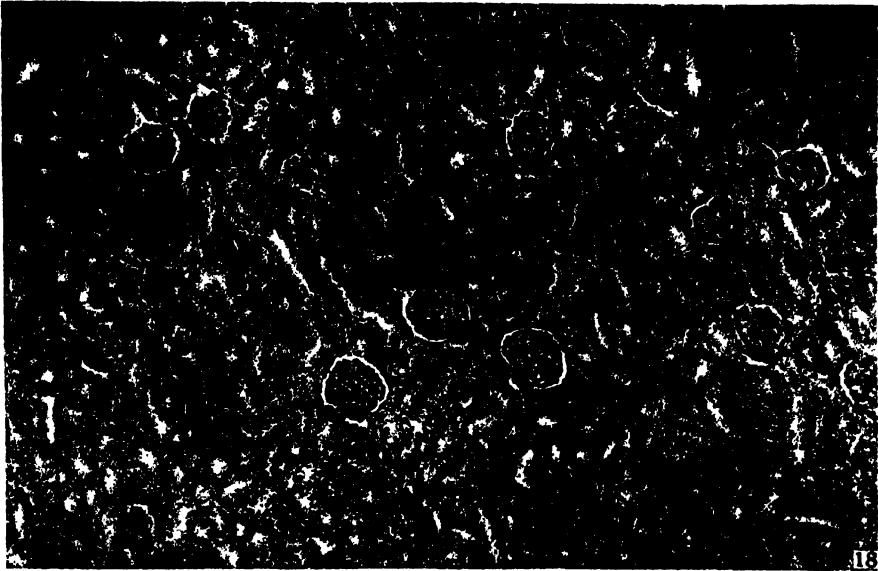
(Pearce: Klosiella Infection of the Guinea Pig.)
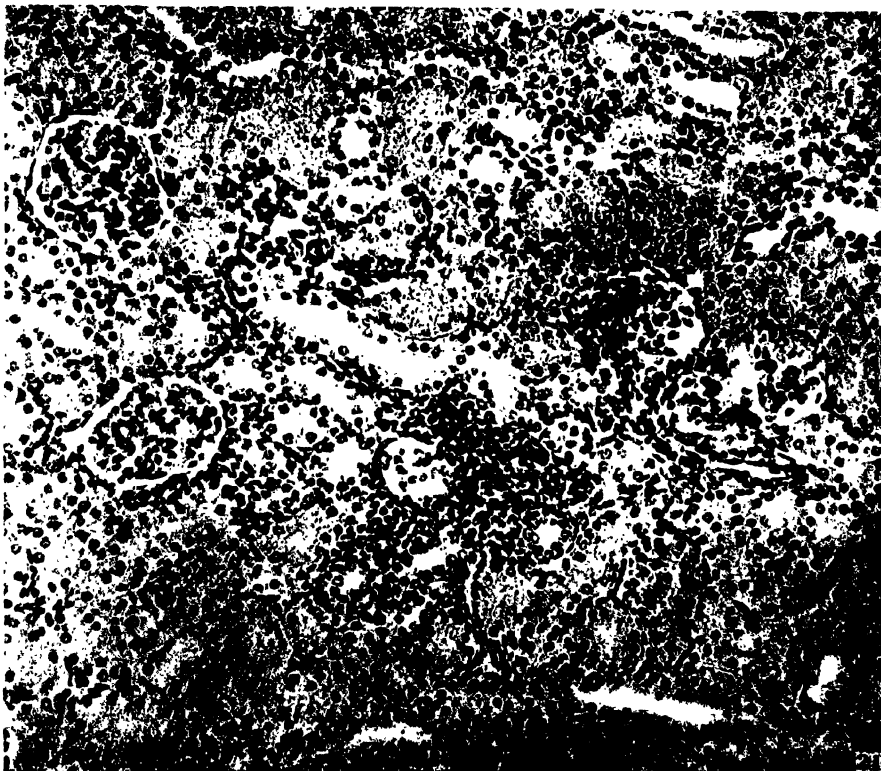
(Pearce: Klosiella Infection of the Guinea Pig.)

CHEMOPATHOLOGICAL STUDIES WITH COMPOUNDS OF ARSENIC.

IV. THE CHARACTER AND DISTRIBUTION OF RENAL INJURY PRODUCED BY ARSENICALS AS INDICATED BY THE PROCESSES OF REPAIR.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 71 TO 76.

In previous papers[1] attention was directed to some of the striking differences in the gross and histological changes produced in the kidneys of dogs by lethal doses of certain compounds of arsenic. Upon the basis of these observations it was pointed out that arsenicals could produce a so called tubular as well as a vascular injury of the kidney and that innumerable combinations of these two fundamental forms of tissue injury were obtainable through the use of arsenical compounds of different chemical constitution.

In order to obtain additional information as to the character and location of the specific renal injury produced by these arsenicals, as well as a knowledge of the subsequent processes of organic repair, we have extended our experiments to a study of tissue changes in the kidneys of animals given sublethal doses of these substances.

EXPERIMENTAL.

In the following experiments guinea pigs were used, as in our experience the kidneys of these animals are as free from spontaneous lesions as those of any animal available for the purpose. However, it should be mentioned in this connection that in the kidneys of some guinea pigs we have found certain pathological changes that seem to be associ-

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[1] Pearce, L., and Brown, W. H., *Jour. Exper. Med.*, 1915, xxii, 517, 525.

ated with the presence of a renal parasite.[2]   The relative importance of this finding in interpreting the various lesions in the processes of repair will be considered in the discussion.

In our previous experiments[1] dogs were used because the comparatively large size of their kidneys showed gross changes advantageously.   However, as we stated then, the histological changes were controlled by experiments carried out with guinea pigs.   The gross and microscopic changes in the kidneys of both dogs and guinea pigs following lethal and sublethal doses of these arsenicals were practically identical with but one exception, that of arsenophenylglycine, to which we shall refer at greater length in discussing the results obtained with this compound.

Sterile solutions of arsenious acid, arsenic acid, sodium cacodylate, salvarsan, neosalvarsan, arsacetin, arsenophenylglycine, and atoxyl were injected intraperitoneally into adult male guinea pigs.   With each compound, one sublethal dose was given and the animal killed after 2 to 5 days.   In addition, with the exception of the salvarsan experiment, other guinea pigs were given two to five small doses of the drug and killed in 3 to 19 days.   In a few instances the animal died within 24 hours after receiving a repeated dose.   In these guinea pigs an acute lesion complicates the one of longer duration.   We were guided in the determination of the size of the sublethal dose by our knowledge of the character and extent of the renal injury caused by lethal amounts of the arsenical in question.   We tried always to inject a non-fatal dose, yet one large enough to cause definite renal injury.

Arsenious Acid.

The acute injury to the kidney produced by large doses of arsenious acid is primarily one of congestion and dilatation of the blood vessels with hemorrhage; the tubular epithelium is degenerated with occasional slight cell necrosis and usually there is a voluminous granular precipitate in the glomerular capsule and the tubules.   The restoration to normal after a moderate dose of arsenious acid is very rapid and there is but slight evidence of the previous injury (Fig. 1). This consists of large, rather irregular glomeruli with somewhat

[2] Pearce, L., *Jour. Exper. Med.*, 1916, xxiii, 431.

shrunken tufts and a distinct although slight increase in tuft nuclei. About the base of the glomeruli are small accumulations of fibroblasts and round cells (Fig. 1) which occasionally slightly invade the adjacent labyrinthine tissue.   Nowhere have we seen any distortion or alteration in the usual architecture in either the cortex or the medulla. The tubular epithelium shows only slight degenerative changes with no evidences of tubular necrosis or regeneration.

Practically the same microscopic picture is found in Guinea Pigs 1, 2, 3, and 4 of this series (Table I), irrespective of the difference in size and number of doses given or the length of survival of the animal.   The changes in Guinea Pig 2 are relatively more marked than in the others, although they are essentially of the same type.

TABLE I.

Arsenious Acid.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.					Doses.	Days survived.	Termination.
	1	2	5	6	11			
1	5					1	3	Killed.
2	3	3		10		3	8	"
3	10		10		10	3	12	Died.
4	8		5			2	6	"

The proliferative changes in the kidney, therefore, after sublethal injections of arsenious acid are extremely slight and are confined to slight increase of nuclei in a somewhat shrunken glomerular tuft and to a slight accumulation of fibroblasts and round cells about the base of the glomeruli.   There are no mitoses in the tubular epithelium, indicating a previous injury of this tissue with subsequent regeneration.

Arsenic · Acid.

The gross and microscopic pictures of the kidneys of guinea pigs poisoned with lethal doses of arsenic acid are practically the same as those of arsenious acid.   Vascular injury is the predominating change and there is but slight tubular necrosis.   However, the study of the kidneys of guinea pigs that have received sublethal doses of arsenic

acid reveals characteristic changes that differentiate them rather sharply from those of arsenious acid. In the inner half of the cortex and in the boundary zone there is a considerable amount of young fibroblastic (connective) tissue extending for the most part along the medullary rays, but also invading the labyrinth to an appreciable degree. Some of the straight and convoluted tubules in these areas are compressed, but many more are dilated and may contain a granular precipitate (Fig. 2). The epithelium of some of these tubules shows degeneration and is flattened and cuboidal in type. In others it is basophilic and evidently of recent formation. Occasionally mitoses are seen. A few of the tubules in these areas appear as more or less solid cords of recently formed epithelial cells, which stain an intense blue with hematoxylin. The glomeruli in the kidneys of this series are practically normal.

TABLE II.

Arsenic Acid.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.							Doses.	Days survived.	Termination.
	1	2	3	5	6	8	10			
2	2		4		4	10	10	5	13	Killed.
3	8							1	6	"
6	10	10		10				.3	7	"

In Guinea Pig 2 (Table II), which received five doses of arsenic acid and survived 13 days, many of the blood vessels in the boundary zone are somewhat thickened. In certain glomeruli some of the capillary walls are apparently thickened also, but this is such an irregular finding and one that may be so variously interpreted that we hesitate to lay much emphasis upon it. The fibroblastic proliferation in the inner cortex and boundary zone is quite characteristic in this animal (Fig. 2). The amount of connective tissue proliferation in Guinea Pig 3 is less than in Guinea Pig 2, but the location and arrangement is the same. In Guinea Pig 6, on the other hand, there is slightly more connective tissue than in Guinea Pig 2, which may be due to the fact that Guinea Pig 6 survived the administration of three comparatively large doses of arsenic acid for 7 days.

In contrasting arsenious and arsenic acid, which resemble each other so closely in the character of the acute renal lesion, it appears that there are distinct differences in their action which are revealed in the process of repair. With arsenious acid, proliferative changes are almost wanting and are practically exclusively confined to the slight increase in nuclei of the glomerular tuft and to slight fibroblastic accumulation about the base of the glomeruli. With arsenic acid, on the other hand, proliferative activity of fibroblasts is appreciably more marked, and is associated with distortion of the tubules especially in the inner half of the cortex and boundary zone and with distinct (although slight) regeneration of tubular epithelium.

Sodium Cacodylate.

TABLE III.

Sodium Cacodylate.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.				Doses.	Days survived	Termination.
	1	2	5	6			
2	50	100		1,000	3	7	Died.
3	400		1,000		2	6	"
4	700				1	2	Killed.
5	700		700		2	6	Died.
6	500	500	.		2	3	"

The kidneys of guinea pigs poisoned with lethal doses of sodium cacodylate belong to the red type and resemble those of arsenious and arsenic acid. In the kidneys of guinea pigs which have survived 2 to 7 days, as is shown in Table III, we find practically the same slight proliferative activity that was observed in the animals which received sublethal doses of arsenious acid. There are occasional small accumulations of fibroblasts and round cells about the glomeruli, along the medullary rays, and in the boundary zone (Fig. 4). In some of the glomerular tufts there is a distinct increase in leukocytes of the endothelial type. There is nothing comparable to the fibroblastic proliferation with consequent distortion of tubules that was comparatively conspicuous in the animals poisoned with arsenic acid. However, in addition to the accumulation of fibroblasts there is more

necrosis of the tubular epithelium than with either arsenious or arsenic acid, and mitoses may be found in both the convoluted and the straight tubules.

There is but little difference, histologically, in the kidneys of the various guinea pigs of this series (Table III). In Guinea Pig 2 there are perhaps more and slightly larger accumulations of fibroblasts. Mitoses are found in the tubular epithelium of all five animals, but they are more numerous in Guinea Pig 6.

There is scarcely any fibroblastic proliferation, therefore, in the animals poisoned with sublethal doses of sodium cacodylate as well as with arsenious acid,—in this respect differing from those poisoned with arsenic acid. But, in addition, there is distinct reparative activity on the part of the tubular epithelium, indicating that this tissue has been injured by the drug, and in this respect differing from guinea pigs poisoned with sublethal doses of both arsenious and arsenic acids, in which the tubular epithelium is but little affected.

Salvarsan.

We have but one instance in which a sublethal dose of salvarsan was given and the animal allowed to live for more than 24 hours. This guinea pig received 150 mg. of the drug per kilo of body weight and was killed 48 hours after its administration.

The acute salvarsan kidney is a red kidney. In this one example of an early stage in the process of repair the glomeruli are large and rather irregular. The tufts on the whole are not shrunken and there is an accumulation of endothelial leukocytes in some of them. Many of the walls of the tuft capillaries are hyaline and distinctly thickened. About the base of some of the glomeruli there is a collection of fibroblasts and round cells which in some instances extends slightly outward into the labyrinth between the convoluted tubules. In the boundary zone and along the inner portion of the medullary rays are small irregular patches of young fibroblasts (Fig. 5), but there is no distortion of the tubular structures. The tubular epithelium is markedly degenerated but there is no definite epithelial necrosis, and since we have seen no mitoses we may infer that the tubular epithelium was not seriously injured. It should be stated in this

connection, however, that this animal received the dose of salvarsan intraperitoneally and that the compound was absorbed relatively slowly, for when the animal was killed 2 days after injection, the abdominal cavity contained a quantity of unabsorbed drug.

The evidences of repair, seen in the kidneys of guinea pigs after salvarsan injection, are therefore chiefly the changes in the glomeruli and the irregular proliferation of fibroblasts about the glomeruli and in the boundary zone.

Neosalvarsan.

The acute neosalvarsan kidney resembles those of arsenious acid and salvarsan. In Guinea Pig 1, killed on the 2nd day of survival, as shown in Table IV, we find definite signs of a proliferative activity

TABLE IV.

Neosalvarsan.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.		Doses.	Days survived.	Termination.
	1	5			
1	300		1	2	Killed.
2	190	500	2	6	Died.

in the kidney in addition to the acute lesion which is still present. About the base of the glomeruli are small accumulations of fibroblasts and round cells. In addition there is a slight diffuse infiltration of the same character along the medullary rays in both cortex and medulla which in some areas invades the adjacent labyrinthine tissue. The glomeruli themselves are extremely irregular; some are of normal size, while others are markedly shrunken with a contracted tuft filling approximately three-quarters of the capsular space. The tubular epithelium is considerably degenerated and in the inner portion of the cortex there is some individual cell disintegration of the epithelium of the loops of Henle and a slight degree of necrosis. A few mitotic figures are seen.

The changes in Guinea Pig 2 are of the same general character as those in Guinea Pig 1, although of a lesser degree. This may be

due to the much smaller initial dose.   The capsules of Bowman and the capillary walls of some of the glomerular tufts are slightly hyaline and thickened.   There are small, irregular patches of fibroblasts and round cells about the glomeruli, along the medullary rays, and in the boundary zone.   The tubular epithelium is degenerated with occasional slight necrosis.   No mitoses are seen.

The changes in the process of repair after neosalvarsan resemble in general those after salvarsan, differing only in the greater irregularity of the glomeruli and the somewhat greater degree of tubular necrosis which is most marked in the inner portion of the cortex. After both salvarsan and neosalvarsan there is a patchy irregular proliferation of fibroblasts in the boundary zone and cortex, but there is no distortion of the tubules as with arsenic acid.

Arsacetin.

TABLE V.

Arsacetin.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.				Doses.	Days survived.	Termination.
	1	5	13	16			
1	125	250			2	6	Killed.
2	100	200	200	200	4	19	"
3	300				1	2	"

The kidneys of dogs and guinea pigs which have received lethal doses of arsacetin are pale and show a most extensive tubular necrosis with a subordinate vascular injury.   In the reaction from a non-fatal dose of this compound there is an exceedingly active and prompt regeneration of the epithelium, especially in the loops of Henle, and to a somewhat less extent in the convoluted tubules and the tubules of the medulla (Figs. 6, 7, and 8).   The glomerular capillaries are somewhat dilated and there may be a slight and irregular accumulation of fibroblasts and polyblasts about the base of the glomeruli, along the medullary rays, and in the boundary zone.   In Guinea Pig 3 (Table V), killed on the 2nd day, there is already marked activity of the tubular epithelium throughout the cortex and to a less extent in

the medulla. There is practically no vascular alteration and no fibro-blastic proliferation in this animal. Guinea Pig 1 was killed on the 6th day after having received two doses of arsacetin. The kidney sections of this animal show a moderate regeneration of the tubular epithelium, almost exclusively confined to the loops of Henle and the medullary tubules. About the base of some of the glomeruli there is a very slight degree of fibroblastic proliferation. Four moderate sized doses of arsacetin were given to Guinea Pig 2 in 16 days, and the animal was killed on the 19th day (Table V). In this animal the regenerative activity of the tubular epithelium has reached a remark-able degree. Mitotic figures are found in practically every microscopic field (4 mm. Zeiss objective; No. 4 ocular) and they are particularly numerous in the straight tubules of the cortex and medulla (Figs. 6, 7, and 8). Many of the tubules along the medullary rays show entirely new epithelium and some of these tubules are almost solid masses of new cells. Degeneration with some necrosis and desquamation of cells is still present in some of the convoluted tubules and limbs of Henle and there are many hyaline casts in the boundary zone and medulla. In the boundary zone, along the medullary rays, and about the base of some of the glomeruli there is a rather diffuse proliferation of fibroblasts and infiltration of polyblasts which irregularly invade the labyrinth in some areas. Several of the renal tubules of this animal contain the parasite which we have referred to and which must be taken into consideration in interpreting the proliferative changes about the glomeruli.

Atoxyl.

The acute lesion in the kidneys of dogs and guinea pigs poisoned with lethal amounts of atoxyl is predominantly tubular, but the vas-cular changes are by no means inconspicuous. In guinea pigs which have received sublethal doses of atoxyl, there is a rapid attempt at regeneration of the tubular epithelium which is shown in both animals of this series (Table VI). Many of the straight tubules are dilated and lined with low cuboidal basophilic staining cells (Fig. 9), and there are numerous mitoses in both cortical and medullary epithelium (Figs. 9, 10, and 11). There is a well marked leukocytic exudate

in the interstitial tissue as well as in some of the tubules of Guinea Pig 3 (Fig. 11); it is less prominent in Guinea Pig 1.   In addition to the marked epithelial necrosis with regeneration and the cellular exudate caused by the injection of atoxyl, there is a definite fibroblastic proliferation along the medullary rays, in the boundary zone, and in the upper medulla (Figs. 11 and 12).

The territorial distribution of fibrosblastic proliferation in Guinea Pig 3 corresponds with that in Guinea Pig 1.   The process, however, is distinctly more pronounced, as might be expected, the animal having received three doses of atoxyl in 8 days.   The fibroblasts are somewhat diffusely distributed throughout the lower edge of the cortex, and along the boundary zone and upper medulla.   Some of the tubules in the area of fibroblastic proliferation are compressed,

TABLE VI.

Atoxyl.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.			Doses.	Days survived.	Termination.
	1	2	5			
1	100			1	3	Killed.
3	50	50	50	3	8	"

while others are slightly dilated (Figs. 9 and 12).   The glomeruli are irregular in both animals.   Some are quite large, others are shrunken.   Many of the tuft capillaries are dilated and the walls moderately thickened.   About the base of the glomeruli are slight fibroblastic accumulations which in a few areas invade the adjacent labyrinth.

The process of repair in the kidneys of guinea pigs poisoned with sublethal doses of atoxyl resembles that of arsacetin in the prompt and marked regeneration of tubular epithelium.   Moreover, there is a definite proliferation of fibroblasts which recalls the changes caused by arsenic acid, and in addition there is a well marked exudation of polymorphonuclear leukocytes into the interstitial tissue and tubules.

Arsenophenylglycine.

Arsenophenylglycine acts somewhat differently in guinea pigs and in dogs.   With lethal amounts of the drug the kidneys of dogs and guinea pigs are both pale, with a predominant tubular necrosis and a relatively subordinate vascular injury.   In the guinea pig, however, after a comparable sublethal dose of the compound there is but little tubular necrosis and but slight vascular injury.   Consequently, after such a dose of arsenophenylglycine the process of repair in the kidney consists only in recovery from a parenchymatous and fatty degeneration of the tubular epithelium with now and then a mitotic figure.

TABLE VII.

Arsenophenylglycine.

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.		Doses.	Days survived.	Termination.
	1	5			
1	250	.	1	2	Killed.
2	250		1	5	"
3	100	50	2	6	Died.
4	400		1	3	Killed.
5	300	300	2	6	Died.
6	500		1	4	Killed.

Such a course of events is followed after a single or a repeated sublethal dose (Table VII).   In Guinea Pig 1, for instance, killed on the 2nd day, degeneration of tubular epithelium is marked, but there is practically no cell necrosis, and no mitotic figures are seen. Guinea Pig 2 received the same amount of arsenophenylglycine as Guinea Pig 1, but was not killed until the 5th day.   The epithelial changes in the two animals are almost identical.   In Guinea Pig 2 there is an increase of tuft nuclei, mostly of the polymorphonuclear variety, and about the base of some of the glomeruli are slight accumulations of fibroblasts.   Guinea Pig 3 received two small doses of the compound, and the alterations here are much the same as in the first two animals.   The leukocytic cells infiltrating the glomerular

tuft are mostly eosinophilic; there is a very irregular thickening of the wall of the glomerular capillaries and an extremely irregular and slight distribution of round cells and fibroblasts about the base of some of the glomeruli, along the straight vessels, and in the labyrinth. The tubular epithelium is swollen and granular and there is some necrosis of a disintegrative character in the epithelium of the outer cortex. An occasional mitotic figure is seen.

The acute changes in the kidneys of guinea pigs after sublethal doses of arsenophenylglycine consist almost entirely of parenchymatous and fatty degeneration of the tubular epithelium with but little cell necrosis, differing in this respect from a similar sublethal injury in the kidneys of dogs. The processes of repair after such an injury are, therefore, comparatively simple and do not involve any appreciable degree of cell regeneration. There is only a slight and very irregular interstitial fibroblastic proliferation.

DISCUSSION.

A study of the processes of repair in the kidneys of guinea pigs poisoned with sublethal doses of certain arsenical compounds furnishes additional information as to the character and location of the acute injury. The idea, which we have previously suggested, namely, that all arsenicals do not produce a purely vascular type of renal injury, is further substantiated by this series of experiments in which the regeneration of tubular epithelium plays a relatively conspicuous part. The participation of the epithelial tissue, however, is by no means the dominant feature in the recovery after sublethal doses of all arsenical compounds, but only after certain particular ones. In others, the acute injury is mainly vascular, and the reaction of the epithelial structures is but slight. Further, the interstitial proliferation of fibroblasts which occurs in a marked degree after injections of various arsenicals may be especially pronounced in the repair following a drug which, in lethal doses, causes but little vascular disturbance, as, for instance, arsacetin. Here, the initial and dominant injury is epithelial; there is only slight demonstrable alteration of the vascular structures. In the process of recovery, however, after the administration of sublethal doses of arsacetin, there may be quite

a marked proliferation of young fibroblasts diffusely distributed (Table V, Guinea Pig 2, and Fig. 8). This is also seen in the repair of the kidney following injections of atoxyl, although here the picture is more complicated, for atoxyl affects the vascular as well as the epithelial tissue of the kidney. The distribution of the proliferating fibroblasts after a more purely vascular injury such as that produced by arsenious or arsenic acid is confined more sharply to the boundary zone, with radiations along the medullary rays to the glomeruli. On the other hand, after arsacetin, the fibroblastic proliferation is more diffusely distributed throughout the cortex, although it may be more numerous in the boundary zone and along the medullary rays.

Taking all these observations into consideration, therefore, it would seem that a toxic agent like arsenic, may, in one or another of its various combinations, injure the vascular, epithelial, or interstitial (connective) tissue of the kidney, if we may judge of such an initial injury by the subsequent processes of repair. The relative distribution and extent of the initial injury may be difficult to determine until one studies various stages of the recovery of the kidney, in which the injured tissues are regenerating. This is particularly true in the case of injury of the connective tissue of the kidney.

After a sublethal dose of arsenious acid which produces an almost pure type of vascular injury in the kidneys, the return to normal is very rapid and there is only a slight fibroblastic proliferation about the base of the glomeruli to indicate a previous injury. Injections of sublethal amounts of salvarsan also cause a proliferation of fibroblasts of a more interstitial and rather patchy character, somewhat greater in extent and amount than with arsenious acid. On the other hand, with arsenic acid, there is a relatively large amount of fibroblastic proliferation, especially in the boundary zone, resulting in the compression or dilatation of some of the tubules, and in addition a slight although definite regeneration of tubular epithelium. Sodium cacodylate in sublethal amounts causes essentially the same slight fibroblastic changes seen after arsenious acid, but a well marked regeneration of tubular epithelium as well. With all these four compounds, arsenious and arsenic acid, salvarsan, and sodium cacodylate, there are slight but fairly regular glomerular changes, consisting of

a slightly swollen tuft and an increase in the tuft nuclei. With neosalvarsan, however, the glomeruli are extremely irregular, some being very large, others contracted and shrunken. There is a slight interstitial fibroblastic proliferation, comparable to that of salvarsan and a slight regeneration of tubular epithelium. After sublethal injections of atoxyl, there is very marked regenerative activity of the tubular epithelium, a leukocytic exudate, and a definite and rather diffuse interstitial proliferation of fibroblasts with dilatation or compression of adjacent tubules. Following a sublethal injection of arsacetin, there is a conspicuous and prompt regeneration of tubular epithelium and a relatively diffuse interstitial proliferation of fibroblasts. Unfortunately, we must omit the experiments of arsenophenylglycine on the guinea pig in this consideration, for the reasons stated above.

It would seem, therefore, that arsenicals which produce an injury that is primarily vascular may lead to only a slight subsequent proliferation of tissue (arsenious acid). However, other arsenicals which produce an acute injury that is vascular, may lead to distinct interstitial proliferation (arsenic acid, salvarsan, and neosalvarsan). Moreover, an arsenical that produces an acute injury that is primarily vascular may also produce injury of the tubular epithelium which in the stages of repair dominates the picture to the exclusion of any extensive interstitial proliferation (sodium cacodylate).

On the other hand, arsenicals that produce primarily an injury of tubular epithelium cause a marked subsequent regeneration of this tissue, and may cause in addition an interstitial fibroblastic proliferation (arsacetin). But an arsenical compound that produces a marked vascular injury in addition to the picture of tubular necrosis may lead to a relatively marked interstitial proliferation as well as the extensive epithelial regeneration (atoxyl).

In connection with the fibroblastic proliferation described in the processes of repair in these kidneys, we must refer to the presence of a parasite in the kidneys of some guinea pigs.[2] This parasite is only occasionally found in guinea pigs, but -we believe that it may cause irregular accumulations of fibroblasts and round cells about the base of some of the glomeruli and in the neighboring labyrinthine tissue. These accumulations resemble those seen after sublethal injection

of arsenious acid, but they are much more irregular. Therefore, we wish to be particularly cautious in a final interpretation of our results and to take into consideration the possibility that some of the fibroblastic proliferation we have seen in the kidneys of guinea pigs after sublethal injections of these arsenicals may be due in part to this renal parasite.

We have shown that after acute lethal injury with various arsenicals, two types of kidneys could be distinguished, both grossly and histologically, namely, the red and the pale. Further, in the reaction after a sublethal injury inflicted with these compounds these two main types of kidney alteration may still be differentiated by the processes of repair. The administration of those compounds which produce a predominantly pale kidney is followed by a most prompt and pronounced regeneration of tubular epithelium with a varying degree of diffuse fibroblastic proliferation. The compounds that produce a predominantly red kidney are followed by proliferation of fibroblasts with but slight regeneration of the tubular epithelium. The distribution of the fibroblasts in the reparative stages apparently corresponds in some degree to the initial injury. After a more or less predominant vascular injury the fibroblasts are usually found about the glomeruli, along the medullary rays, and in the boundary zone. With other compounds that show little evidence of an acute vascular injury, the connective tissue injury may be more pronounced, and in recovery from such an injury the fibroblasts are diffusely distributed throughout the cortex and medulla in a typical interstitial manner. But, just as there are innumerable combinations of vascular and tubular and probably connective tissue injury in the acute arsenic kidneys, so there are innumerable combinations of the different tissue elements in the processes of repair. Hence, we suggest that the character and distribution of renal injury produced by arsenical compounds as indicated by the processes of repair are bound up in the chemical constitution of these compounds. Further, that each particular compound as far as the kidney is concerned, acts as a more or less specific toxic agent, as shown by the character and distribution of the renal lesions.

SUMMARY.

1. The processes of repair in the kidneys of guinea pigs after sublethal doses of certain arsenical compounds indicate that all arsenicals do not produce a purely vascular type of renal injury.

2. While some arsenicals produce a predominantly vascular injury and others produce a predominantly tubular injury, both these tissue elements are undoubtedly always affected, although in varying proportion. In addition, the interstitial connective tissue is probably always affected. The diffuse proliferation of this tissue may be relatively conspicuous in the processes of repair after arsenicals that cause but slight vascular injury.

3. All red kidneys do not necessarily show identical pictures during the processes of repair; the same is true of pale kidneys.

4. The mode of action of an arsenical compound as a renal toxic agent is bound up with the chemical constitution of the compound.

EXPLANATION OF PLATES.

The illustrations are all from untouched microphotographs.

PLATE 71.

Fig. 1. Arsenious acid. Guinea Pig 2. Section from the outer cortex. There is a regular and definite although slight accumulation of round cells and fibroblasts about the glomeruli. The nuclei of the glomerular tuft show a slight increase. The tubular epithelium is normal. ×·210.

Fig. 2. Arsenic acid. Guinea Pig 2. Section from the inner cortex. There is a slight interstitial proliferation of connective tissue, and the renal tubules in this area are somewhat distorted, some being dilated and others compressed. The tubular epithelium and glomeruli are practically normal. × 210.

PLATE 72.

Fig. 3. Arsenic acid. Guinea Pig 6. Section from the boundary zone. There is a small but conspicuous proliferation of connective tissue with consequent dilatation and compression of the renal tubules in this area. The renal epithelium is swollen and shows parenchymatous degeneration. × 210.

Fig. 4. Sodium cacodylate. Guinea Pig 2. Section from the outer cortex. The glomerular tuft is slightly swollen and shows a slight increase of tuft nuclei. There is a definite and regular although slight infiltration of round cells and fibroblasts around the base of the glomeruli. The renal epithelium is slightly swollen and degenerated. × 210.
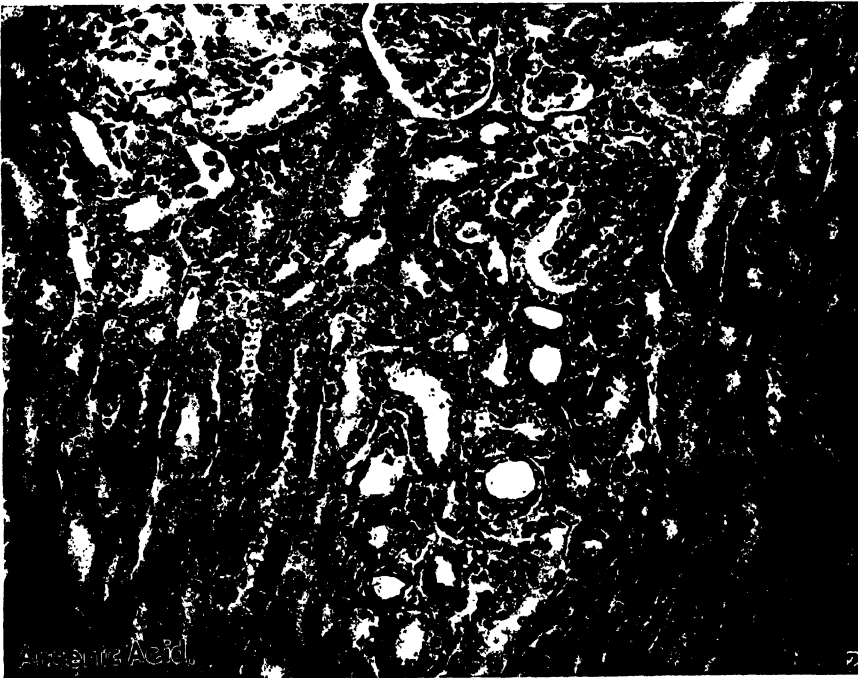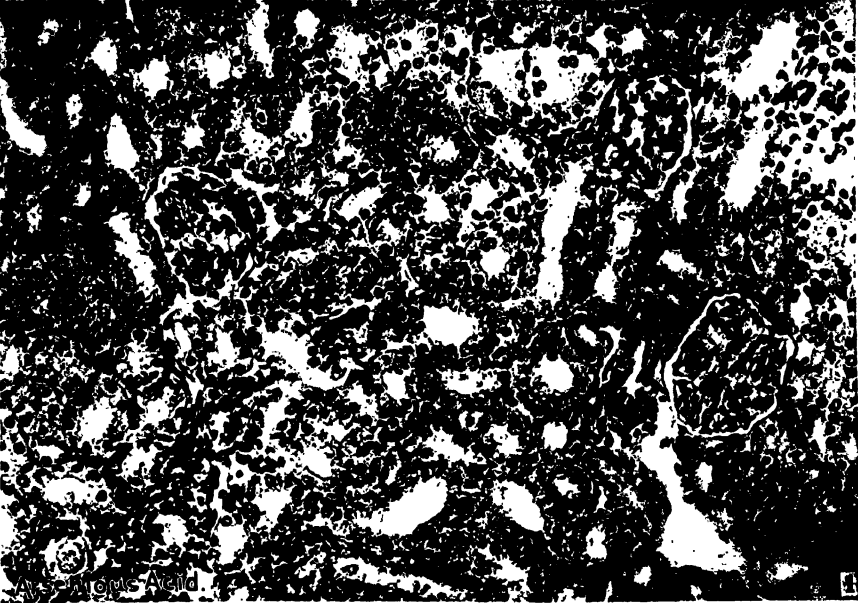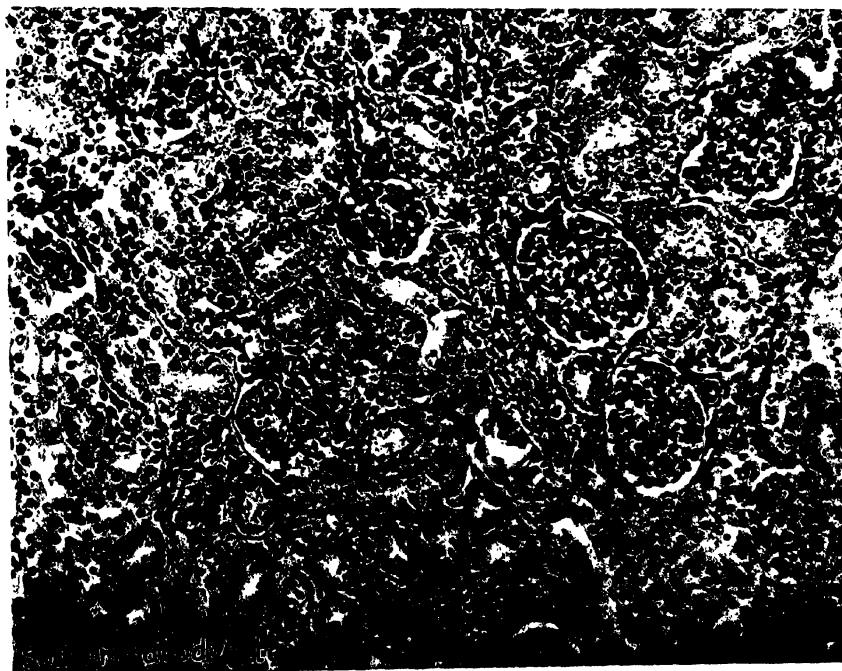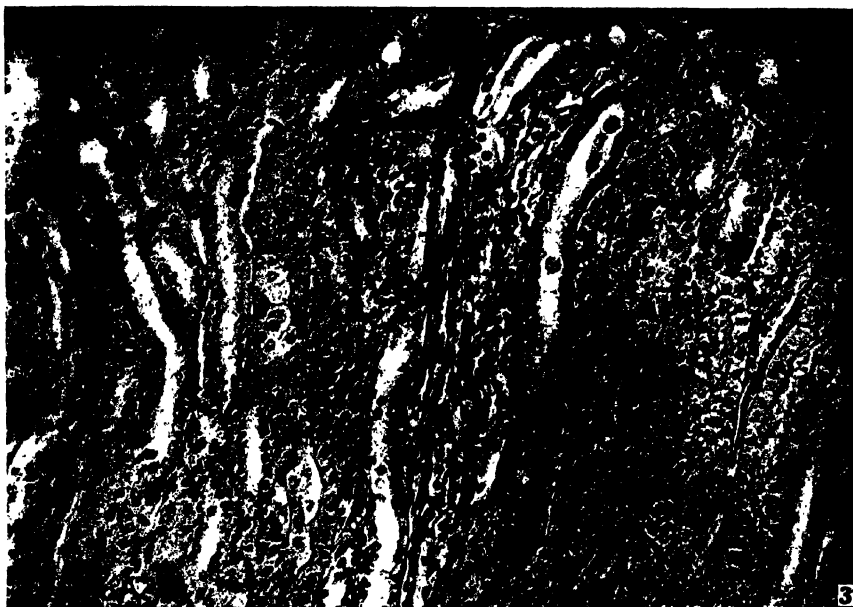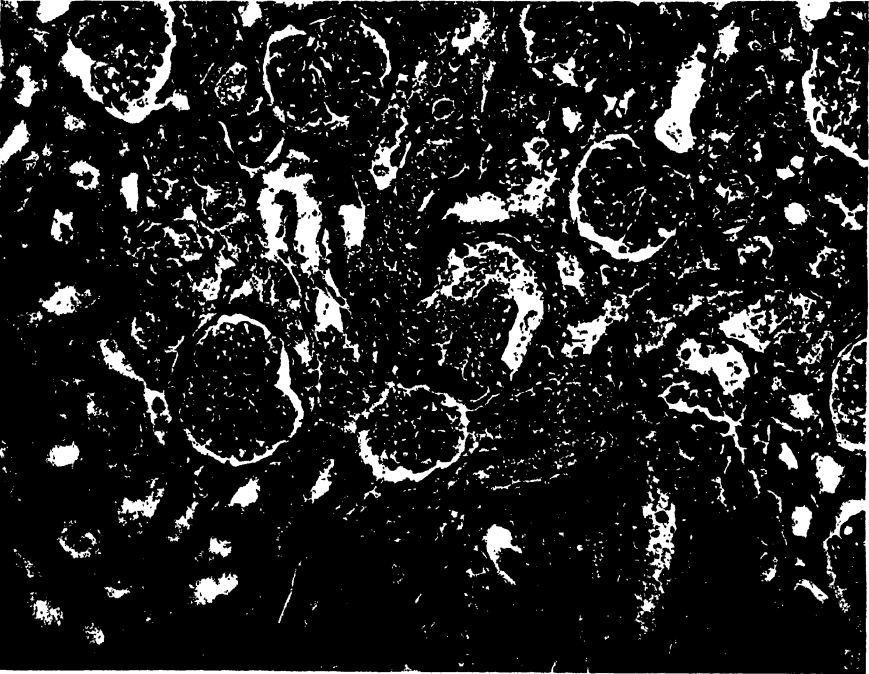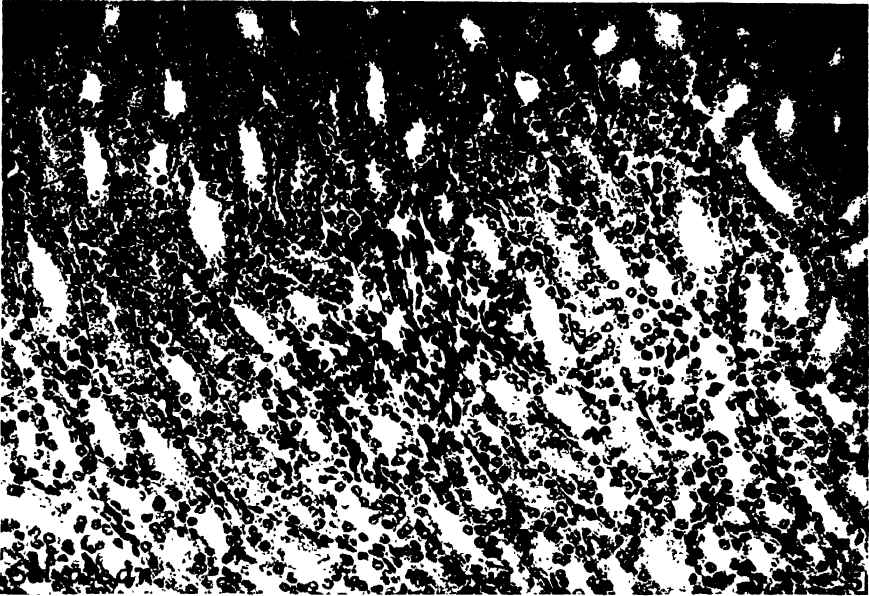
PLATE 72.

(Pearce and Brown: Renal Injury and Repair.)

PLATE 73.

FIG. 5. Salvarsan. Guinea Pig 1. Section from the boundary zone. There is an irregular patchy proliferation of connective tissue. The renal epithelium is practically normal. × 210.

FIG. 6. Arsacetin. Guinea Pig 2. Section from the outer cortex. There is a marked degeneration and regeneration of the tubular epithelium with numerous mitotic figures. There is a granular precipitate in the lumen of the tubules which appear slightly dilated. The glomeruli are normal in appearance. There is a slight diffuse proliferation of fibroblasts. × 210.

PLATE 74.

FIG. 7. Arsacetin. Guinea Pig 3. Section from the midcortex. There is a moderate degeneration and marked regeneration of the tubular epithelium with numerous mitotic figures. There is a granular precipitate in the lumen of the tubules. × 210.

FIG. 8. Arsacetin. Guinea Pig 2. Section from the upper medulla. There is marked regeneration of the tubular epithelium with numerous mitotic figures. There is a slight but definite interstitial proliferation of fibroblasts. × 210.

PLATE 75.

FIG. 9. Atoxyl. Guinea Pig 3. Section from the inner cortex. There is a conspicuous dilatation of some of the tubules with a granular precipitate in the lumen. The tubular epithelium shows regeneration with numerous mitotic figures. The glomeruli appear normal. × 210.

FIG. 10. Atoxyl. Guinea Pig 1. Section from the outer cortex. There are two adjacent mitotic figures in the epithelium of a convoluted tubule. × 1,470

PLATE 76.

FIG. 11. Atoxyl. Guinea Pig 3. Section from the boundary zone. There is a conspicuous leukocytic infiltration in the tubules and interstitial tissue. The tubular epithelium shows numerous mitotic figures. There is some interstitial proliferation of the connective tissue. × 378

FIG. 12. Atoxyl. Guinea Pig 3. Section from the boundary zone. There is a diffuse interstitial proliferation of connective tissue. The tubular epithelium shows several mitotic figures. × 378.

A METHOD FOR OBTAINING SUSPENSIONS OF LIVING CELLS FROM THE FIXED TISSUES, AND FOR THE PLATING OUT OF INDIVIDUAL CELLS.

BY PEYTON ROUS, M.D., AND F. S. JONES, V.M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 84 TO 87.

The only cells of the mammalian body which lend themselves as individuals to accurate experimentation *in vitro* while yet alive are the blood cells, the cells of exudates, and the spermatozoa. In saying this we do not overlook the usefulness of tissue cultivation or of experiments with living tissue fragments, of the transplantable tumors for instance. But both means of study involve, not individual cells, but complexes of different cells, which can be standardized only roughly, and which cannot be broken up into their component elements or protected from confusing factors, such, for example, as are introduced by death and autolysis of the central tissue portions. These difficulties have led us to work out a method whereby living tissue cells can be obtained as individuals in suspension, and, if desired, can be plated out in a culture medium (plasma) just as are bacteria. After growth the cells can be liberated again, and again plated successfully.

The method consists, in brief, in the growth of tissue in plasma, according to Carrel and Burrow's modification of Harrison's technique, and the liberation of the new cells by digestion of the clot with trypsin. We had noted that if the serum of a growing tissue culture is replaced with Locke's solution at room temperature the cells of the growing strands that extend out into the medium sometimes contract into spheres, which may be separate or, when growth has been dense, loosely attached, side by side. The general outline of the culture is maintained because the cells are held in place by the fibrin network; and if serum is added and incubation renewed they again put forth processes, and, joining each other, again form strands. The problem

has been to cause the cells to contract and then to liberate them from the fibrin network. This is readily done with trypsin in Locke's solution (Fig. 1); and the resulting suspension can be freed by filtration of all but individual cells.

Method.

We have used the trypsin powders of Merck, Grübler, and Kahlbaum. It is necessary to free them as far as possible from the ammonium sulphate which constitutes the greater part of their bulk. According to Kirchheim,[1] the trypsin of Merck does not contain ammonium sulphate; but we have found it present in as great amount as in the other preparations mentioned. It should be got rid of by Kirchheim's method. The trypsin powder is shaken briefly in absolute alcohol and allowed to stand while the heavy sulphate settles out. The supernatant flocculus is collected on a filter, rapidly washed with ether, dried in the air, and dissolved in Locke's solution (Locke's modification of Ringer's solution, but without sugar). The yield from 2 gm. of the unpurified trypsin is dissolved in 98 cc. of Locke's solution. The cloudy, yellowish fluid is filtered, first through paper, then through a Berkefeld cylinder (N) to sterilize it, and is distributed in test-tubes and kept in the ice box. It loses very slowly its ability to digest and can still be used after 2 months. 3 per cent trypsin digests plasma clots more rapidly and does not harm most cells; but 5 per cent kills cells. Unpurified trypsin powders can be employed but the results are not so good.

The tissue from which cells are to be obtained should be cultivated preferably in plasma diluted with Locke's solution in order that the fibrin network to be digested shall be slight. A mixture of one part of plasma with three of Locke's solution is a medium suitable for most tissues. If there is need for a thick suspension of cells many bits of tissue should be grown. It is convenient to flood them in small Petri dishes with a thin layer of the dilute plasma. After clotting has taken place each dish is sealed to prevent evaporation, and placed in the incubator. A stout cord dipped in hot, sterile paraffin and thrust between the outer and inner rim of the dish, with one end

[1] Kirchheim, L., *Arch. f. exper. Path. u. Pharm.*, 1911, lxvi, 352.

left free, is useful for sealing. A pull on the free end will release the top of the dish.

When growth is established the trypsin solution, warmed to 37°C., is poured on, filling the dish above the plasma, and incubation is continued. In a few minutes some of the tissue fragments are free, and within about an hour the clot has disappeared and there remains a clear fluid containing numerous tissue particles. This is taken up with a pipette, stirred to break up any loose aggregations of cells, diluted with Locke's solution, filtered through sterile gauze, and centrifugalized. The fine, powdery, yellowish gray sediment will consist of discrete cells, nearly all of them alive. They can be washed repeatedly if need be. We prefer for this purpose the "gelatin-Locke's,"—Locke's solution containing ⅛ per cent of gelatin,— which, as Rous and Turner showed,[2] protects fragile cells against mechanical injury. If the cells are to be plated again in plasma they need not be washed, but after centrifugalization can be suspended in the Locke's solution used to dilute the plasma. Plating is done, as before, in Petri dishes.

Results.

The cells liberated as individuals by trypsin are those which grow out into the medium in strands or a meshwork, or which wander out separately (connective tissue cells, endothelium (?), choroid, sarcoma, and splenic tissue cells). Thus far we have used successfully the tissue of rat and chick embryos, of rat and chicken tumors, and the normal tissue of young rats. Sheets of growing cells (epithelium) are not readily broken up. Whether individual epithelial cells can be liberated in this way is as yet uncertain. But small groups of epithelial cells are obtained, and bits of striated muscle which live for a brief period when plated again.

The individual cells become approximately spherical when in suspension and the nuclei also tend to, though less perfectly. The change in form is especially noteworthy in the case of elements which, when growing in culture, are stellate or of an attenuated spindle shape with an elongated nucleus. When freed, suspended in

[2] Rous, P., and Turner, J. R., *Jour. Exper. Med.*, 1916, xxiii, 219.

serum, and stained, such cells show no trace of the long protoplasmic processes which they had while growing. With Wright's stain certain of them derived from connective tissue and probably of fibroblastic and endothelial origin have a resemblance to the mononuclear series of the blood (Fig. 2). Their cytoplasm is basophilic. Other cells from the same source are three or four times the diameter of any blood element. These morphological features will be taken up in a later paper.

The freed cells, distributed in plasma as separate individuals and incubated, soon put forth processes and assume their original form. Bits of striped muscle from the embryo may round at the ends, thus gaining a leech shape, and put out short processes (Fig. 4). We have not observed them to proliferate. But the spindle-shaped and stellate cells of connective tissue, sarcoma, and the choroid coat of the eye multiply rather rapidly. If the cells are numerous the plate will show at the end of 24 hours a thick mesh- or feltwork consisting of elements once separate which have reached out and joined each other by means of attenuated processes (Fig. 3). The tendency of scattered cells thus to connect with each other again is striking. At the end of 48 hours the number of growing elements is greatly increased, not only by proliferation but by the "waking up" of cells previously spherical. If small masses of cells are present in the culture, as the result of incorrect filtration, growth from them may be almost explosive, each mass resolving itself into elements that radiate in every direction.

The Replating of Cultures.

The limits of the method have not yet been reached. The freed and plated cells can be liberated anew after growth and successfully plated again in fresh plasma. To judge from our results, the process can be repeated indefinitely. Isolated cells of the chick's choroid continue to form pigment after they have been twice liberated with trypsin and twice replated (Fig. 5).

Cells that have been growing in tissue cultures for more than 24 hours when freed and examined in suspension show, as a rule, fat droplets, and corresponding vacuoles when fixed and stained in the

spherical state. Fat droplets have often been noted in tissue cultures and their source is to some extent known.[8] But they are much less prominent in the culture with its extended cells than in the freed, contracted elements. We wish to emphasize the fact that they develop very early, even when growth is taking place in a dilute plasma medium. Only during the first 24, or rarely the first 48 hours, do the cells appear absolutely normal. Later the culture consists for the most part of abnormal elements. This is true also of the freed and plated cells. It follows that replating should be carried out at least every 48 hours.

Technical Difficulties.

The initial cultures must be free from bacteria if the cells are to be replated after their liberation. For the tryptic digestion liberates not only tissue cells but bacterial colonies, and a single one of these latter can by its dispersion ruin all of the new plates. For this reason it is best to cut up the tissue to be grown, in a sterile, glass-sided box, closed with pieces of rubber dam at the ends, through apertures in which the instruments and tissue are introduced, and the hands thrust, encased in sterile, rubber gloves. A small, glass hood with cloth sides will do nearly as well, and it is useful for the replating of cultures. Needless to say a single contamination at any time will ruin a sequence of plates. If the cells are to be used in suspension it is of less importance.

The centrifugalization to bring down tissue cells brings down also fine débris such as bits of cotton, particles of dust, etc., from the fluid. By the time cultures have been twice digested and plated, enough of this will have been collected to mar their appearance, unless special care is taken. Such care consists in the use of well filtered fluids, and centrifuge tubes closed with corks instead of cotton or gauze stoppers. Much time can be saved if the corks are hollowed to fit over the end of the tube, but with a central core to. prevent dislodgement (Text-fig. 1). They may be boiled or autoclaved. The central core should be rather short in order that it may remain uncontaminated when the cork is placed on an unsterile surface.

[8] Lambert, R. A., *Jour. Exper. Med.*, 1914, xix, 398.
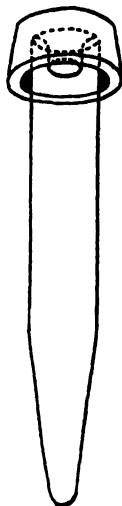
TEXT-FIG. 1. Centrifuge tube closed with an easily removable cork designed to keep the contents sterile.

SUMMARY.

Individual, living, tissue cells can be obtained in suspension by digesting with trypsin the clot of growing tissue cultures. Under these circumstances the living cells assume a spherical form. When washed and plated in fresh plasma they put out processes and proliferate. After growth in the new plates has occurred the digestion and plating can be repeated. The limits of the method have not yet been reached. We are at work on a number of the problems which it has opened up.

EXPLANATION OF PLATES.

PLATE 84.

FIG. 1. Edge of a culture undergoing digestion with trypsin. The cells have begun to contract into spheres. (Chick embryo.)

PLATE 85.

FIG. 2. Connective tissue and endothelial (?) cells liberated from cultures of the heart muscle and abdominal muscle of a 3 day old rat. Mononuclear cells from the blood of the same animal. Wright's stain. All the cells are drawn to the same magnification.

Fig. 1.

Blood

Mononuclears   Small Series   Large Series   Muscle

Heart   Large Series
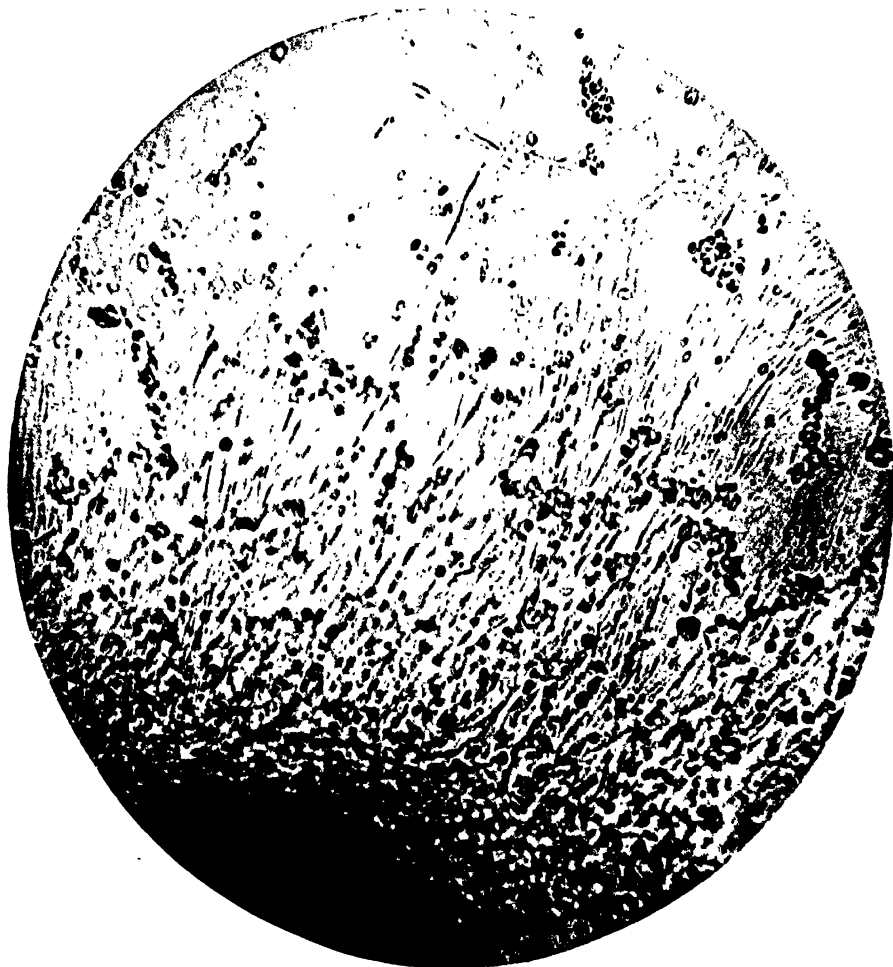
FIG. 3

Fig. 4.

Fig. 5.

(Rous and Jones: Living Cells from Fixed Tissues.)

The cell marked *a* has ingested two red cells. One cell of the muscle series shows vacuoles resulting from a fatty change, and another has attached to it undigested fibrin threads.

PLATE 86.

FIG. 3. Meshwork formed by the anastomosis of connective tissue cells liberated by trypsin and plated as separate individuals. (Chick embryo.)

PLATE 87.

FIG. 4. Striped muscle from a culture incubated 24 hours after liberation by trypsin and replating.

One fragment of muscle, with sharp-cut ends has not grown and has undergone fatty change. But the others give evidence of life, as shown by their change in form, and one has put forth a process. (Rat embryo.)

FIG. 5. Cells from the chick's choroid growing after two liberations with trypsin and two replatings. The formation of pigment is going on actively.

THE PROTECTION OF PATHOGENIC MICROORGANISMS BY LIVING TISSUE CELLS.

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PLATE 90.

The discovery by Metchnikoff of the purposeful character of phagocytosis has so stimulated investigation of the defensive activities of tissue cells that phagocytosis and bacterial destruction are at present almost synonymous in the general mind. The old view that leukocytes provide ingested organisms with a culture medium and a means of transport is now mentioned only in historical résumés. It is known that bacteria may be ingested alive—Metchnikoff himself utilizes this fact in his demonstration of the importance of cells for immunity[1] —and it is known also that a cell may take up too many microorganisms and dying of a surfeit, as one might say, may fail to kill them. But such occurrences are regarded as mere incidents in the process of destruction. The possibility that in certain instances cells not only fail to kill the organisms they ingest, but actively protect them from circulating antibodies seems not to have been considered. Yet the question thus raised has more than passing interest. There are a number of important diseases, among them leprosy, tuberculosis, gonorrhea, Leishmania, caused by microbic parasites which live more or less habitually within tissue cells. The part played by the host cells in the life of such microorganisms and also in the distribution within the body of the diseases they induce has obvious importance.

Unfortunately it is not possible to make direct *in vitro* tests with the microorganisms mentioned and the cells in which they live, for the reason that they fail to give rise to circulating antibodies active

[1] Metchnikoff, E., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 679.

enough to be suitable for the tests. But the problem can be approached by means of artificial systems. It is possible, for example, to submit leukocytes that have ingested bacteria to a bactericidal serum and observe its effect on the intracellular organisms.

Protection against a Foreign Antiserum.

In a first experiment we have used *Bacillus typhosus*, the leukocytes of the guinea pig, which, according to Pettersson,[2] contain no substances destructive to typhoid bacilli, and the serum of normal rabbits, which is strongly bactericidal for the organism.

Experiment 1.—Washed leukocytes from two sterile, 18 hour, aleuronat exudates of the guinea pig's peritoneal cavity were made into a single thick suspension with Locke's solution, and incubated with typhoid bacilli in the presence of a much diluted mixture of fresh guinea pig serum and antityphoid rabbit serum. The smallest amount of the mixture that would ensure good phagocytosis had been previously determined. Twice this amount was employed. The suspension of typhoid bacilli consisted of four 24 hour slant agar cultures of different strains[3] made up in 80 cc. of Locke's solution.

After 1 hour's incubation films from the phagocytic mixture and from a control mixture without serum were examined, with Manson's stain. Only in the former was phagocytosis observed. It was profuse, though there were still many free bacteria. Now small portions of the mixtures and of other control mixtures were added to large amounts of fresh, normal rabbit serum, and the incubation was continued 2 hours longer. Plating was then done in equal portions of agar. Duplicate tests were made throughout. For the dilutions Locke's solution was used.

As the experiment shows. leukocytes can protect ingested bacilli from the action of a bactericidal antiserum.

The mixture of leukocytes and bacteria subjected to preliminary incubation without serum (Mixture 2), and consequently free of phagocytosis at the end of the first hour, gave many more colonies in the plates made 2 hours after the addition of rabbit serum than did Mixtures 3 and 4 from which leukocytes were absent. This was probably due to the protection of ingested organisms, despite the fact

[2] Pettersson, A., *Centralbl. f. Bakteriol.*, *1te Abt.*, *Orig.*, 1905, xxxix, 423.

[3] These were laboratory strains known respectively as Board of Health, Metchnikoff, Wassermann, and New York Hospital, which had been under cultivation for more than 2 years.

TABLE I.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	cc.	cc.	cc.	cc.	1 hr.'s incubation 0.3 cc. from each tube was mixed with 2.4 cc. normal rabbit serum; and after 2 hrs.' more incubation 0.5 cc. of this mixture was plated in 6 cc. agar.		Same as A except that 0.3 cc. was mixed with 2.4 cc. Locke's solution instead of serum.	
1	—	0.2	0.2	0.1	0.1		211 294		About 500.
2	0.2	0.2	0.2	—	—		72 49		Innumerable.
3	0.2	—	0.2	0.1	0.1		10 13		Exceedingly numerous.
4	0.4	—	0.2	—	—		25 27		Innumerable.

that the bacteria were all outside the cells at the time the rabbit serum was added. For the rabbit serum itself was able to cause phagocytosis and did actually cause this in the mixture, as the films show. Presumably it brought about the ingestion of some living bacteria which were then protected from its further action by the cells containing them.

The character of the protection was not determined in this experiment. The results of Table I can be interpreted otherwise than wholly in terms of bactericidal action. The serum was agglutinative; and agglutination can of itself produce a reduction in the number of colonies from a bacterial suspension. The leukocytes might have protected the bacilli merely mechanically against clumping and thus have brought about the results seen in the plates. Obviously, for further work a non-agglutinating bactericidal *agent was desirable.*

An Indicator of Cell Death.

The question came up, furthermore, whether the leukocytes exerting a protective influence were alive. Rabbit serum contains an hemolysin for guinea pig erythrocytes, and might well kill the white cells of this species. To solve the point resort was had to tests with

trypan-blue. Evans and Winternitz[4] state that the dye rapidly colors the nuclei of dead cells but does not stain living ones. The following experiment confirms their observation.

Experiment 2.—A 4 day aleuronat exudate from the pleural cavity of a dog was washed and suspended in Locke's solution. It contained many large mononuclear cells capable of phagocyting rat erythrocytes. A part of the suspension was mixed with rat erythrocytes and dog serum, and incubated for 1 hour, after which an equal bulk of a freshly prepared and filtered solution of trypan-blue (0.02 gm. in 2.0 cc. of Locke's solution) was added and the cells forthwith examined. The nuclei of the majority of them failed to stain. None of the many cells that had phagocyted erythrocytes showed nuclear staining.

Other portions of the original suspension were kept in the ice box for several days and then treated in the same way. Most of the cells now failed to take up the rat corpuscles, and most stained promptly with trypan-blue. Among the few which did not stain were those which had just phagocyted rat cells.

The results of this experiment have been borne out by many subsequent observations involving injury to cells of other types, among them the cells liberated from tissue cultures by digestion of the plasma clot with trypsin.[5] Trypan-blue is a prompt and reliable indicator of whether cells are alive or dead.

Protection against an Inorganic Disinfectant.

The test with trypan-blue showed that rabbit serum is injurious to guinea pig leukocytes subjected to it under the conditions of Experiment 1. At the end of 2 hours' incubation about half the leukocytes were dead, as shown by the nuclear staining, whereas in control specimens incubated in salt solution, they were nearly all alive, very few stained cells being observed. Because of the unfitness of rabbit serum for our work, as thus manifested, it was necessary to find another bactericidal agent, one that would not harm the leukocytes or agglutinate the bacteria. Potassium cyanide proved to have both these qualities.

Clowes[6] was the first to demonstrate the difference in resistance of tissue cells and bacteria to potassium cyanide. As far as we are

[4] Evans, H. M., and Winternitz, M. C., unpublished work, cited by Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443.

[5] Rous, P., and Jones, F. S., *Jour. Exper. Med.*, 1916, xxiii, 549.

[6] Clowes, G. H. A., *Brit. Med. Jour.*, 1906, ii, 1548.

aware, his important observations have not been followed up. He found that tumor cells treated *in vitro* with cyanide in a concentration that killed bacteria remained capable of causing tumors on implantation. Our tests have shown that $\frac{N}{150}$ potassium cyanide in Locke's solution will destroy typhoid bacilli in heavy suspension while failing to kill guinea pig leukocytes, as shown by the trypanblue test. Furthermore, the bacilli are not agglutinated. In Experiment 3 advantage has been taken of these facts.

Experiment 3.—This experiment closely followed Experiment 1 except that potassium cyanide was substituted for rabbit serum. A watery solution of potassium cyanide, isotonic with 0.95 per cent sodium chloride, was used, diluted with Locke's solution to $\frac{N}{100}$ concentration.

No phagocytosis was observed in Tube 2 after the preliminary incubation; but it was pronounced in Tube 1, though large numbers of bacilli were still free. The leukocytes were tested with trypan-blue at the time of plating and were found to be, in general, still alive, as proved by the fact that their nuclei failed to stain. In more concentrated potassium cyanide solutions they died early as shown by the stain. Some potassium cyanide was carried over into the agar plates but there it was greatly diluted. Control tests with regard to this point showed that the addition to agar of more than ten times the amount of potassium cyanide present in our plates failed to prevent or even to delay the growth of typhoid organisms.

TABLE II.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	ſc.	cc.	cc.	cc.				
1	—	0.2	0.2	0.1	0.1	1 hr.'s incubation then 1.2 cc. $\frac{N}{100}$ potassium cyanide added; 2 hrs.' more incubation and 0.2 cc. plated with 6.0 cc. agar.	144 142	Same as A except that 1.2 cc. Locke's solution added instead of the potassium cyanide.	About 1,500
2	0.2	0.2	0.2	—	—		2 2		Innumerable.
3	0.2	—	0.2	0.1	0.1		0 0		About 1,500
4	0.4	—	0.2	—	—		0 0		Innumerable.

Here there was a marked protection of bacteria by the tissue cells containing them (Table II).   The potassium cyanide entirely sterilized the suspensions in which the bacilli were free, whereas in the mixtures in which phagocytosis had occurred, many bacilli survived its action and grew in the plates.   One or two colonies developed from Mixture 2, in which there was, supposedly, no phagocytosis because of the absence of serum.   But, as is well known, phagocytosis takes place to a slight extent in the absence of serum.   The result is readily explained on this basis.

Despite the results of this experiment, the evidence for a protection exerted by living cells specifically is not conclusive.   For had the cyanide killed but one phagocyte in every several hundred,—and this may well have happened,—the gross number would be sufficient to permit of an interpretation of the results in terms of a protection exerted by dead phagocytes, not by living ones.

Protection against an Homologous Antiserum.

A second objection to experiments such as the foregoing is that they have little in common with the conditions in the animal body. We have sought to meet both these difficulties by employing red cells as the test object, placing phagocytes which contain them in an hemolytic antiserum derived from an animal of the sort furnishing the phagocytes.   With such material it is easy to follow the fate of the ingested corpuscles and to determine by means of the trypan-blue stain whether the individual phagocytes exerting protection are alive or dead.   And if the time element be disregarded, one can with good reason liken the conditions as regards the ingested rat corpuscles to those affecting pathogenic microorganisms existing within cells bathed with a lymph containing antibodies.

We have employed rat red cells allowing large mononuclear cells from an exudate in the dog's pleural cavity to phagocyte them, after which an anti-rat dog serum has been added to the mixture.   In order to follow the fate of the ingested red cells it has been necessary to know what changes they would undergo when injured within the phagocytes by the hemolytic serum.   For the corpuscles cannot lake as they would when free.   Fortunately these changes proved easily

recognizable when several red cells were present side by side in a single phagocyte. They have been described and figured by Levaditi[7] and others, who noted them, as we have done, within phagocytes that had taken red cells out of an hemolytic mixture after the hemolysin had attached itself. Under such circumstances hemolysis goes on within the phagocyte with the result that the included corpuscles melt together, as it were, forming one or more large, orange-yellow hemoglobin-containing globules, which on pressure escape from the cell to dissolve instantly in the surrounding fluid. The contrast between these intracellular globules and red cells that remain intact after ingestion is pronounced (Fig. 1).

Experiment 4.—The phagocytes used were mononuclear cells of a 4 day aleuronat exudate in the pleural cavity of a dog. The exudate was washed twice and made into a thick suspension. It contained a very few red cells. The opsonizing serum was derived from the same dog, and so too the 25 per cent suspension of washed dog cells needed for the controls. The serum hemolytic for rat cells came from another dog which received three intravenous injections of rat erythrocytes on successive days and was bled 8 days thereafter. Preliminary examination of the two dogs' blood had shown that they did not agglutinate or hemolyze each other.

The form of the experiment was simple. Rat cells, dog exudate, and a little normal dog serum were mixed together, and, when phagocytosis had taken place, anti-rat dog serum was added to some portions of the mixture, and to others an equal quantity of Locke's solution. Incubation was resumed and from time to time the phagocyted red cells were observed for evidence of destruction. Two preliminary tests were necessary.

(*A*) A determination of the least amount of dog serum which would incite to phagocytosis. The dog serum used for its opsonins contained, as is usual, an hemolysin for rat cells. But the test showed that it could be used to incite phagocytosis in an amount far below the one producing visible erythrocytic change.

(*B*) A test to find out how much anti-rat dog serum was required to hemolyze free rat cells so rapidly that they could not be taken up by dog phagocytes mixed with them. For the anti-rat serum was not only hemolytic but an active stimulant to phagocytosis as well. And, had it been added to the phagocytic mixtures in a quantity which permitted the taking up of cells while hemolysis of them was going on, a proper comparison between the corpuscles in the phagocytes submitted to serum and those submitted to Locke's solution would have been impossible. The results of this test were so interesting that they will be given in detail.

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[7] Levaditi, C., *Ann. de l'Inst. Pasteur*, 1902, xvi, 233.

TABLE III.

Mix-ture.	25 per cent rat red blood corpus-cles.	Dog serum.	Exu-date.	Anti-rat serum.	Apparent hemolysis.	Real condition as determined microscopically.
	cc.	cc.	cc.	cc.		
1	0.1	0.1	0.25	0.5	Complete within 5 min.	No phagocytosis. Complete hemolysis.
2	0.1	0.25	0.25	0.25	Complete. (?)	Considerable phagocytosis. All free red blood corpuscles hemolyzed.
3	0.1	0.25	0.25	0.25 of 50% solution.	+++	Profuse red sediment, of phagocytes enormously distended with red cells. All free red cells hemolyzed.
4	0.1	0.25	0.25	0.25 of 25% solution.	++	

Incubation was for 1 hour at 37°C.

Only in the first mixture was there complete hemolysis and in this all the red cells had suffered destruction within the first 5 minutes of incubation (Table III). In the other mixtures the degree of color of the supernatant fluid at the end of an hour indicated incomplete hemolysis as did the profuse red sediment. But these findings were not due to the serum's lack of hemolytic power, for, as the microscope showed, all the red cells remaining free had been hemolyzed. Many though, had been ingested by cells of the exudate, and thus were protected from hemolysis. The abundant red sediment consisted of phagocytes distended with red cells. Some of the phagocytes had extended only the thinnest layer of glassy cytoplasm over the red corpuscles which stood out, quite unhemolyzed, as knobs on their surface. Such corpuscles were evidently protected from the serum by their intracellular situation. But most of the ingested red cells had been much injured and had coalesced into orange-yellow globules (Fig. 1).

The test made it evident that in order to avoid phagocytosis in the presence of the hemolytic serum sufficient of this serum must be added to cause hemolysis of all the red cells within 5 minutes.

Now the main experiment was proceeded to. The following mixtures were made in a number of tubes.

Mixture 1.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 25 per cent dog serum.

Mixture 2.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 10 per cent dog serum.

At the end of an hour's incubation the mononuclear cells were found to have ingested numbers of apparently unchanged red cells,—from 6 to 25, as a rule.

To some of the duplicate tubes 0.5 cc. anti-rat dog serum was added, to others the same quantity of Locke's solution, and incubation was resumed. At the end of a further hour the preparations were examined for evidence of destruction of the intracellular red cells. None had occurred. The erythrocytes within the mononuclears submitted to anti-rat serum, like those within the phagocytes treated with Locke's solution, were still intact (Fig. 2). But the anti-rat serum had hemolyzed all extracellular erythrocytes and the phagocytes lay in the midst of masses of shadows.

A still more exacting test was carried out, as follows:

Mixture 3.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. concentrated dog serum. After 1 hour's incubation 0.5 cc. anti-rat serum was added to some of the tubes, an equivalent amount of Locke's solution to others, and incubation continued for 2 hours more. In this instance the amount of serum used for opsonization caused injury to the red cells, of which some coalesced into globules immediately after their ingestion, though the majority remained intact. And now when the phagocytes full of these globules and of more or less injured and vulnerable cells were submitted to anti-rat serum and to Locke's solution, respectively, for 2 hours, no microscopic differences in their contents were observed. The anti-rat serum had been powerless to enhance the breaking-down of the red cells.

The dog red cells present in the exudate were far too few to constitute a source of error in the findings. Nevertheless, a number of control tests were made with a 25 per cent suspension of dog cells instead of rat cells. They were not phagocyted. In the experimental tests proper, the free dog cells were easily distinguished by their failure to agglutinate or hemolyze in the anti-rat serum.

The results of this experiment were clear-cut. The phagocytes protected red cells within them from the action of a powerful homologous antiserum (Figs. 2 and 3).

Protection a Function of the Living Cell.

The condition of the leukocytes exerting this protective action remained to be determined. Were they perhaps injured by the antiserum, despite the absence from it of agglutinins and hemolysins? The failure of the serum to penetrate could be explained in this way. Or was the protection a function of the living leukocytes and of living ones only? Tests with the material of Experiment 4 threw light on these points.

Experiment 5.—(A) Cells of the ultimate mixtures of Experiment 4 were examined with trypan-blue. The phagocytes which had been incubated with

anti-rat serum and those submitted to Locke's solution alike failed to take the stain. Many of the white cells that had failed to ingest red corpuscles showed nuclear staining.

(*B*) The following mixture was made up with the ingredients of Experiment 4:

0.1 cc. Locke's solution + 0.5 cc. washed exudate + 0.5 cc. concentrated anti-rat serum + 0.5 cc. dog serum.

After 1 hour's incubation the cells were separated out with the centrifuge and made up as follows:

0.5 cc. treated cells + 0.1 cc. 25 per cent suspension of red blood corpuscles + 0.5 cc. dog serum.

At the end of an hour profuse phagocytosis had taken place, proving that the leukocytes could not have been seriously injured by the antiserum.

(*C*) Portions of the ultimate mixtures of Experiment 4, in which phagocytosis had occurred, were kept in the ice box at about 2° C. and examined each day. At the end of the first 24 hours the cellular sediment had largely lost its ruddy color. The microscope showed that this was due to diffusion out of the phago-cytes of pigment from the ingested red cells. The majority of the leukocytes had now a ground glass appearance. In Mixtures 1 and 2 there could be seen within the phagocytes the intact stromata of red cells from which the hemo-globin had disappeared. In Mixture 3 the stromata were not so clearly visible. In this instance, one will recall, the majority of the red cells were much damaged previous to ingestion. In all the mixtures there were still some phagocytes con-taining bright red cells, and in Mixture 3 some with orange-yellow globules. Phagocytes containing one or two intact cells and the shadows of others were not observed; but the protoplasm of many phagocytes was stained light orange, due to the seeping out of the hemoglobin from ingested red elements. With the trypan-blue test it was found that the cells containing bright erythrocytes or globules regularly failed to stain. So too did the cells tinted light orange. Practically all the other leukocytes underwent an immediate nuclear staining.

After 3 days in the ice box the results were identical except that living phago-cytes were now rare.

This experiment proved that the protection exerted by the phago-cytes in Experiment 4 was not due to injury, but on the contrary was associated with active cell life. When the phagocytes died they be-came permeable, allowing a rapid diffusion outwards of the hemo-globin from the ingested erythrocytes, as well as diffusion inwards of the trypan-blue stain. It seems highly probable from these facts, as well as from common knowledge of the differences in permeability between dead and living tissues, that when phagocytes die they must lose largely if not entirely their protective power.

DISCUSSION.

There are in the literature a number of detached observations which corroborate our findings. Bordet found that "cholera spirilla injected into the blood stream of cholera immune animals are taken up by the leukocytes even before they can be subjected to lysis by the circulating lytic antibodies."[8] And Metchnikoff, Levaditi,[7] Briscoe,[9] and others have shown that red cells injected into the previously immunized animal may be phagocyted before they can hemolyze. But so far as we are aware no attention has been paid to these indications of protection by cells. Such protection had no practical importance in the instances cited because the phagocytes themselves were capable of destroying the organisms they had ingested. In our experiments as well the phagocytes may have possessed this ability. The demonstration of protection by them is not thereby invalidated. For the ability of cells to protect ingested organisms from the action of outside agencies must be considered as entirely distinct from the disposition they ultimately make of these organisms.

It remains to be determined how far the protection of microorganisms by living tissue cells, especially cells incapable of killing the microorganisms, is important in disease processes. The phenomenon may have much to do with the survival in the animal body of organisms such as the leprosy bacillus which is so often found living within cells of the fixed tissues; and it may serve to explain in part the therapeutic difficulties in such instances. It may throw light, furthermore, on the formation of new disease foci at points of injury in individuals of high general resistance. For if an infective agent can be "walled off" from the action of the body fluids by the protoplasm of a single cell containing it, there is no reason why it should not be carried unharmed wherever this cell goes.

CONCLUSIONS.

1. Living phagocytes are able to protect ingested organisms from the action of destructive substances in the surrounding fluid, and even from a strong homologous antiserum.

[8] See Zinsser, H., Infection and Resistance, New York, 1914.

[9] Briscoe, J. C., *Jour. Path. and Bacteriol.*, 1908, xii, 66.

2. There is evidence that the protection by phagocytes is largely if not entirely conditioned on their being alive.

3. These facts should be taken into consideration in the study of diseases caused by infectious agents capable of living within tissue cells.

EXPLANATION OF PLATE 90.

FIG. 1.   Red cells hemolyzing within phagocytes.   There is a coalescence of the cells into globules.   × 625.

FIG. 2.   Intact red cells of the rat within dog phagocytes submitted for 1 hour to a powerful anti-rat dog serum.   Many of the red cells appear pale because they are out of focus.   The only free erythrocytes that have not been laked are a few dog cells.   × 625.

FIG. 3.   Red cells of the rat still intact within dog phagocytes submitted for 2 hours to a dog serum strongly hemolytic for rat cells.   The shadows of numerous erythrocytes hemolyzed while free are just visible.   × 625.
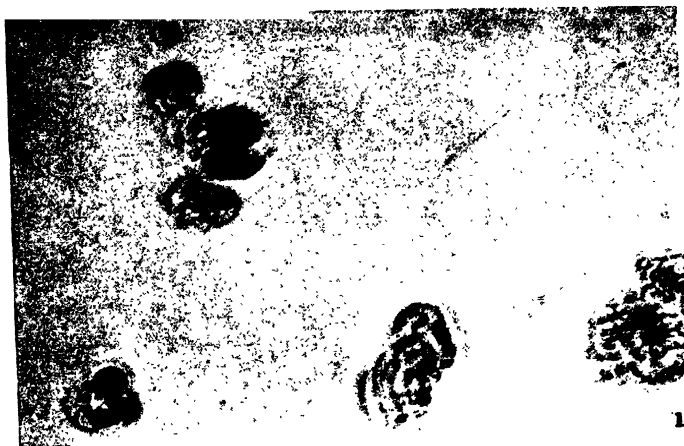
CHEMICAL VERSUS SERUM TREATMENT OF EPIDEMIC MENINGITIS.

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INTRODUCTION.

The reappearance of epidemic meningitis among the belligerent armies in Europe has led to numerous reports on the treatment of the disease. For the most part, the antimeningococcic serum has been employed, but in one instance at least the attempt has been made to improve upon the serum treatment by substituting for it chemical treatment. This attempt to employ directly antiseptic drugs in the treatment of epidemic meningitis is a revival of an old notion.

The circumstances are such as to make the chemical treatment favorable from a theoretical standpoint. The infection tends to be local, that is, within the cerebrospinal membranes, and not general or throughout the body. The membranes are directly accessible from without, so that the chemical substance can be brought into immediate relation with the seat of infection. Moreover, the meningococcus as cultivated outside the body is not a highly resistant microorganism, but is readily injured by chemical action, and is indeed quickly injured by the waste products of its own growth. What, therefore, is apparently simpler or more promising than to control epidemic meningitis by the direct application by means of lumbar puncture of bactericidal chemicals to the infected and inflamed cerebrospinal membranes?

There is, moreover, still another theoretical reason for resorting to the chemical treatment of epidemic meningitis. The meningococcus is now known to be a microorganism which occurs in at least two varieties or types, distinguished as meningococcus and parameningococcus.[1] Their main differences are immunological, so that they

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[1] Amoss, H. L., and Wollstein, M., *Jour. Exper. Med.*, 1916, xxiii, 403.

are subject to therapeutic influences through antisera only when correspondence exists between the infecting type of microorganism and the particular specific antibodies present in a serum. Presumably, chemical agents being less specific would be less restricted in their effects, and might be expected to act quite irrespective of the type of meningococcus causing the infection.

Wolff[2] has recently recommended the employment of protargol in the treatment of epidemic meningitis, basing the recommendation of its use on eight cases, of which five recovered. The point of departure in the selection and administration of protargol was the successful employment of so called colloidal silver preparations in the treatment of gonorrhea, and the close relationships as regards conditions of culture, survival, and reaction to antiseptics subsisting between gonococci and meningococci. It is conceded by Wolff that because of the highly variable clinical course of epidemic meningitis, the number of cases which he reports is too small to permit of the drawing of any conclusions regarding the therapeutic efficiency of the chemical. He believes, however, that it is at least innocuous and can, therefore, be administered with the assurance that if it is not curative, it will do no harm.

The first method of direct chemical treatment of epidemic meningitis to receive considerable attention was that in which lysol was employed. The method was introduced in Lisbon during the epidemic which prevailed there in the first years of the present century, and was briefly reported on by Seager[3] in 1902. Following Seager's report, lysol was tested in the course of the epidemic from which Greater New York was suffering at the time.[4] This epidemic continued for several years and led to the working out of the serum treatment, but at that early date no specific means of combating the disease was known.

It is remarkable and instructive to consider that in these instances, namely, the one relating to lysol and the other to protargol, what appeared to be preliminary success was achieved by the chemical treatment. Wider experience, however, led to the abandonment of

[2] Wolff, G., *Deutsch. med. Wchnschr.*, 1915, xli, 1486.
[3] Seager, H. W., *Lancet*, 1902, ii, 1188.
[4] Manges, M., *Med. News*, 1904, lxxxiv, 913.

the lysol treatment.   In the meantime, knowledge has been gained and methods perfected through which the value of a proposed form of treatment of epidemic meningitis can be determined experimentally in animals, with a degree of accuracy exceeding that based on clinical and limited statistical observations on human beings.

It happens that epidemic meningitis in man is a highly variable disease.   The severity fluctuates between clinical conditions so slight as to justify the term abortive being applied to certain cases, and at other times so severe (fulminant) as to cause death swiftly, and sometimes with the first appearance of symptoms.   Hence the mortality percentage of the disease varies in different epidemics and places. To determine approximately what the effects of varying modes of treatment are it is necessary to compare coincidently treated and untreated cases of sufficient number.   A definite result is therefore obtained only after some time, and by comparing figures obtained in different localities and over a comparatively long period of time.   So long as the figures based on small local observations alone are available, no final deduction can be made.

The employment of animals for the test is free of the uncertainty of the statistical method.   The dosage of the culture of meningococcus can be adjusted to produce the result desired.   Then the effect and the manner of the therapeutic action can be accurately ascertained.   Tests of this kind have been made with protargol and for comparison with lysol and with serum, with results unfavorable to the chemical treatment.

EXPERIMENTAL.

The experimental studies on epidemic meningitis carried out during the past 10 years have shown two kinds of animals to be suitable for determining the pathogenic action of meningococci and the effects of therapeutic agents in meningococcic infection.   The animals are monkeys[5] and young guinea pigs.[6]   While the age of the monkeys seems not to matter, older guinea pigs are highly resistant to meningococcic infection.   The cultures of meningococci are injected into

[5] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 142.
[6] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 168.

the peritoneal cavity in the guinea pigs and into the subarachnoid space in the monkeys, in the latter by means of lumbar puncture. The injected meningococci set up local inflammations in the exudate attending which the fate of the microorganism can be followed precisely, as in human cases of epidemic meningitis in which the cerebrospinal fluid is removed from time to time by lumbar puncture.

The changes occurring in the meninges or the peritoneum vary with the activity and the dose of the culture. Non-virulent cultures are quickly phagocyted, no considerable multiplication takes place, and the inoculated animals show few or no symptoms and recover fully in 24 hours. Active cultures multiply, are imperfectly phagocyted, and cause death according to the virulence and dose in 6 to 8 to 12 hours in guinea pigs, and in 20 to 48 hours in monkeys. At autopsy living meningococci are present in the peritoneal or meningeal exudate and in the blood. Film preparations show meningococci free and phagocyted also in the peritoneal fluid and more numerously in the omentum of the guinea pig, and both free and phagocyted in the exudate in the pia-arachnoid membranes of the monkey.

In carrying out the experiments a virulent strain of the meningococcus (Isadore) was employed. Tests made to determine the optimum period of growth at which to employ the culture indicated it to be after about 18 hours' incubation at 37°C. Sheep serum dextrose agar was used as a medium. Growths 24 hours old infected far more irregularly than those 18 hours old. The reason for the disparity is found in the spontaneous degeneration of the culture, which probably has already begun at 18 hours, and can be detected by microscopic examination at 22 hours. In the guinea pigs it was found in this instance, as already described,[1] that consistent results are best attained by employing one minimal lethal dose of the culture. In this way, lack of protective power is made to assert itself readily, while the existence of protective power is easily demonstrated and can be further investigated. In monkeys the infecting dose is easily determined, although the fatal dose is established less readily. While these animals were used sparingly, the protocol in each instance indicates the outcome of the therapeutic test compared with the control. Besides the result based on death or survival, the changes

taking place in the cerebrospinal fluid as followed by lumbar puncture, and the clinical course of the experimental disease, to a less definite degree, are also valuable indications of the action of the therapeutic agent.

Tests with Guinea Pigs.

The guinea pigs ranged in weight from 90 to 110 gm. The injections were intraperitoneal. The protargol was employed in 0.2 per cent strength, prepared by suspending the dried powder in sterile distilled water. The cultures of meningococci on sheep serum agar after 18 hours' incubation were suspended in sterile salt solution, and injected either immediately or after such intervals as are indicated in the separate experiments. The dose was one m.l.d.

TABLE I.

Experiment 1. Toxicity of Protargol.

Weight of guinea pig.	Quantity of suspension injected.	Result.
gm.		
90	0.3 cc. + 0.7 cc. water.	Survived.
90	0.5 " + 0.5 " "	"
90	1.0 " + 1.0 " "	"
90	2.0 " + 2.0 " "	Died in 38 hrs.

The tests shown in Table I may be taken to indicate that the toxic dose of protargol for young guinea pigs is well above the dose employed for therapeutic purposes.

Experiment 2. Therapeutic Tests of Protargol Intraperitoneally in the Guinea Pig.—One m.l.d. of living culture in a total volume of 1 cc. was injected intraperitoneally into young guinea pigs. The amount of 0.2 per cent protargol suspension given was made up to 1 cc. with sterile water and injected at the time indicated in Table II.

TABLE II.

Weight of guinea pig.	Dose of protargol suspension.	Time of administration.	Result.
gm.	*cc.*		
110	0.5	Immediately.	Death in 12 hrs.
110	0.5	"	" " 21 "
95	0.5	"	" " 15 "
100	0.5	"	" " 10 "
99	0.1	"	" " 8 "
82	0.1	"	" " 19 "
82	0.1	"	" " 11 "
100	0.5	After 15 min.	" " 12 "
110	0.7	" 15 "	" " 8 "
100	0.5	" 1 hr.	" " 10 "
75	0.1	" 1 "	" " 15 "
75	0.1	" 1 "	" " 18 "
95	0.25	Mixed with culture, injected after 1 hr.'s contact.	" " 25 "
110	1.00	" " "	" " 14 "

This experiment is conclusive in demonstrating that protargol in 0.2 per cent suspension, the strength employed by Wolff,[2] is incapable of preventing in guinea pigs the lethal effect of a single minimal fatal dose of the meningococcus, whether administered combined with the culture immediately after the mixture, or whether the culture and protargol are injected separately 15 minutes apart. The period of survival of the treated animals may be about the same as or somewhat greater than the controls. Living meningococci are always found in the peritoneal cavity and in the heart's blood. In this connection it is interesting to find that when the culture and protargol suspension which have been in contact for 1 hour are transplanted to fresh sheep serum agar, no growth is obtained; while, however, cultures from the peritoneal cavity of the dead animals which were inoculated with the mixture are positive, thus indicating that the protargol merely inhibits the growth of the meningococci, but may not destroy them in 1 hour's time. This observation raises the question whether the failure of the protargol is due merely to its imperfect bactericidal power, or whether it depends upon lack of antitoxic power at the same time. The next experiment bears on this consideration.

Experiment 3. Therapeutic Test of Protargol with Autolysate.—An autolysate[7] of the meningococcus was prepared in the usual way by suspending the culture in salt solution, adding toluene, incubating, allowing the toluene to evaporate, and centrifugalizing. The clear supernatant fluid was employed. The autolysate and protargol suspensions were mixed and immediately injected, as shown in Table III.

TABLE III.

Weight of guinea pig.	Dose of autolysate.	Dose of suspension of 0.2% protargol.	Result.
gm.			
90	0.25 cc. in 1 cc. salt solution.	None.	Died in 7 hrs.
100	0.25 " " 1 " " "	0.5 cc.	" during night.
110	0.25 " " 1 " " "	1.0 "	" " "

This experiment indicates that protargol does not exhibit pronounced antitoxic properties, although a delay was noted in the fatal issue in the animals in which it was given.

Mechanism of the Action of Protargol and of Antiserum.

In carrying out the above tests which were made on several different occasions, control observations with antimeningococcic horse serum were also made. The quantity of serum administered was 0.5 cc., and in every one of the six instances in which the antiserum was given the animal survived. The antiserum was also injected in association with the protargol, without, however, preventing a fatal issue. On the other hand, the antiserum influences directly the mechanism of action of protargol, although unable apparently to overcome wholly some injurious effect which it exerts. The next experiment, therefore, was devised to bring out definitely the mechanism of action of the protargol in comparison with the manner of action of the antiserum.

Experiment 4. Mechanism of the Action of Protargol.—Four guinea pigs, ranging in weight from 90 to 110 gm., were employed. Besides the inoculation of meningococci, one of them received immediately 0.5 cc. of a suspension of 0.2 per cent protargol alone; the second received 0.5 cc. of antiserum alone; and

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[7] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 105.

the third 0.5 cc. of a suspension of protargol and 0.5 cc. of antiserum in immediate succession. The fourth animal received only the culture. All were chloroformed at the expiration of 6 hours, at which time the control animal was prostrate and the others were definitely ill.

The autopsies were made at once and the distribution of meningococci in the abdominal cavity studied by means of cultures and film preparations, and in the blood of the heart by cultures alone.

Guinea Pig 1. Protargol Alone.—Two drops of the blood of the heart were laked in 0.5 cc. of sterile water and plated in sheep serum agar. The colonies numbered about 2,000. Two drops of the slightly turbid peritoneal fluid were similarly plated. The colonies numbered about 2,000. The film preparations from the fluid in the abdominal cavity showed numerous scattered meningococci and a notable absence of polynuclear cells, and the omentum showed scattered free meningococci and polynuclear leukocytes, of which a very small number contained diplococci, the leukocytes themselves staining feebly and appearing degenerated.

Guinea Pig 2. Antiserum Alone.—Two drops of the heart's blood were treated as in No. 1. No colonies. Two drops of the somewhat turbid peritoneal fluid gave about 2,500 colonies. Film preparations of the peritoneal fluid showed numerous well preserved polynuclear leukocytes containing many meningococci, while the extracellular diplococci, small in number, were arranged in small clumps. The film preparations from the omentum exhibited many more leukocytes, most of which were filled with meningoccoci in various stages of disintegration, the leukocytes themselves appearing in normal condition. A few extracellular agglutinated meningococci were also present.

Guinea Pig 3. Protargol and Antiserum.—Two drops of the heart's blood yielded one colony, and two drops of the clear peritoneal fluid about 150 colonies. Film preparations of the peritoneal fluid showed a smaller number of diplococci than in either of the preceding animals. Polynuclear leukocytes were present, but in far smaller number than in No. 2, and many were degenerated and had rarely phagocyted the diplococci. Such meningococci as were present at all were agglutinated. The films from the omentum showed somewhat larger numbers of degenerated polynuclear cells, of which a part contained meningococci, and quite numerous free, single and clumped diplococci.

Guinea Pig 4. Control.—Two drops of the heart's blood gave about 2,000 colonies, and two drops of the clear peritoneal fluid innumerable colonies. Congestion of the peritoneal organs was more marked in this animal than in the others. Film preparations of the peritoneal fluid showed a very large number of particularly well preserved meningococci, but no polynuclear leukocytes, and those of the omentum showed a similar condition. The absence of polynuclear cells from the latter structure is particularly noteworthy.

This experiment confirms the observation made upon the guinea pigs treated with protargol which were allowed to succumb. Its

significance is clear.   Protargol has some power to cause dissolution of the meningococci with which it comes in contact, but does not destroy all.   A later increase occurs and invasion of the blood stream takes place.   Probably the injurious action of the protargol arises from its antileukotactic properties, for it causes degeneration of the polynuclear leukocytes and prevents phagocytosis, both *in vivo* and *in vitro*.

This particular action of the protargol is rendered obvious at once on comparison with the manner in which the antiserum acts.   In this instance there is a diversion of polynuclear leukocytes in large numbers to the peritoneal cavity and an acceleration of phagocytosis. Hence at a period at which many living meningococci are still present in the abdominal cavity, practically no escape is taking place into the general blood stream.   The addition of the antiserum to the protargol obviates partially the antileukotactic effect of the latter, but does not completely overcome it, because of the injury inflicted by the chemical on the leukocytes brought out by the antiserum.

Protargol is, therefore, bactericidal for the meningococcus, as for the gonococcus, through direct chemical action, but it is not curative in meningococcic infection in the guinea pig, because being confined within the abdominal cavity it exerts a passive chemotactic and antiphagocytic influence on the polynuclear cells which are themselves injured and destroyed through its action.   Thus the imperfect bactericidal effect is inadequate to control infection, while the antileukotactic action is itself harmful and promoting of infection.   Moreover, the masses of protargol are themselves taken up by leukocytes, to be transported doubtless to organs in which the substance may remain indefinitely.

Tests with Monkeys.

The inoculation of virulent meningococci into the subarachnoid space in monkeys causes an acute inflammation which extends throughout the meninges and into the cerebral ventricles.   Depending upon the pathogenic power and the dose, the animal which develops symptoms within a few hours may die within 24 hours or may recover.   According as one or the other result is obtained, the meningococci multiply and are imperfectly phagocyted, or they increase little

and are perfectly phagocyted.   The cerebrospinal fluid at first remains clear, then becomes turbid with emigrated polynuclear leukocytes; finally it may again become clear.   A blood invasion often follows within a few hours of the inoculation, but is rarely present at the end, even in fatal cases.

Experiment 5.   Control.—A *Macacus rhesus* was injected at 11 a.m. intraspinally with 2 cc. of a salt solution suspension, representing 1½ agar slant cultures of meningococcus, the strain being the same as that used in the experiments on the guinea pigs. 5.00 p.m., animal sick. Lumbar puncture yielded a fluid already turbid, giving positive culture and showing on films many meningococci, few leukocytes, and little phagocytosis. Blood taken from arm vein gave positive cultures. 9.00 a.m. the next day, prostrate. Lumbar puncture yielded highly turbid fluid, giving positive cultures and films in which leukocytes were numerous and of which many contained meningococci; free diplococci were also present. Death at 3.30 p.m. Survived 20½ hours.

Autopsy.—The membranes of the brain and spinal cord showed turbid fluid and congestion. The cultures and film preparations confirmed previous findings. Both intra- and extracellular meningococci were present. Cultures positive. Cultures of heart's blood negative.

Sections from the brain and spinal cord show an acute fibrinopurulent meningitis, most pronounced over the convex surface of the brain. The ventricles are involved slightly. Many diplococci are present in the exudate and are contained chiefly, if not wholly within leukocytes.

Experiment 6.   Treatment with Antimeningococcic Horse Serum.—8.30 a.m., a *Macacus rhesus* received intraspinally an injection of a suspension of meningococci as in Experiment 5. 12.30 p.m., lumbar puncture yielded cloudy fluid, giving positive cultures. Films showed numerous meningococci, pus cells, and phagocytosis already present. The culture from the heart's blood was negative. 2 cc. of antiserum were injected. 4.30 p.m., animal appeared well; lumbar puncture yielded turbid fluid, giving positive culture and showing in the film marked phagocytosis and agglutination of all extracellular meningococci. 8.30 p.m., active. 28 hours after the inoculation the animal appeared well; lumbar puncture yielded a turbid fluid which gave a negative culture. The films showed many leukocytes, some containing meningococci and very few extracellular diplococci. A number of endothelial cells and lymphocytes were present. The animal remained well.

Experiment 7.   Treatment with Protargol.—8.30 a m., a *Macacus rhesus* received an equal part of a suspension of the culture used in the previous experiments. 12.30 p.m., little change in condition. Lumbar puncture yielded a cloudy fluid, from which cultures were positive and films show many leukocytes and well advanced phagocytosis of meningococci; some free meningococci were also present. Injected 2 cc. of 0.2 per cent protargol suspension. 2.30 p.m., animal

lying down, drowsy, surface cold, limbs spastic. 4.00 p.m., respiration rapid, slight convulsion recurring frequently. Blood withdrawn at this time gave a positive culture. 4.30 p.m., died. Survived 8 hours.

Autopsy.—No lesions were found outside the cerebrospinal membranes. The meninges contained an excess of almost clear fluid. No marked congestion. Cultures from the meninges of the brain and spinal cord yielded luxuriant growth. Films showed a large number of meningococci, both intra- and extracellular, and a widespread degeneration and even disintegration of leukocytes. The meningococci stain sharply and deeply. The choroid plexus of the lateral ventricle and the meninges of the olfactory lobes gave results similar to those of the other membranes. Cultures from the heart's blood were positive. Moreover, cultures prepared from the nasal mucosa yielded, among other bacteria, typical meningococci, possessing identical agglutination reactions with the strain employed for inoculation.

The microscopic examination of sections from the central nervous organs shows many pus cells, with here and there red corpuscles, and no fibrin. The number of meningococci present is very large. Some degree of phagocytosis exists; but a striking phenomenon is the packing of lymph spaces with innumerable diplococci. They invade the pial spaces, infiltrate the superficial layers of the cerebral cortex, and follow the perivascular spaces for some distance into the cortex. Certain coagula of red and white corpuscles within the central ventricles also enclose diplococci.

A comparison of the results in the three monkeys inoculated with meningococci and left in one case untreated, and in the others treated either with antiserum or protargol, is unfavorable to the treatment with protargol. The untreated or control animal succumbed under conditions indicating definite if inadequate reaction against and resistance to the infection. The serum-treated animal was already ill when the antiserum was administered, the effect of which was to disperse almost immediately all the symptoms and lead to prompt recovery, coincidently with the removal by phagocytosis of the meningococci. The third monkey exhibited practically no symptoms 4 hours after the inoculation when the protargol was given, but prostration and rapid intensification of symptoms followed almost immediately. Death occurred 4 hours later. The causes of the unfavorable results are to be sought in the two or three main effects of the protargol: (*a*) on the leukocytes, which already present, were greatly injured; (*b*) on the meningococci, which doubtless were also affected through direct bactericidal action from which toxic substances were set free; and (*c*) finally on removal of all restraint to

multiplication of surviving diplococci, since the immense numbers present in the lymph spaces as well as in the exudate indicate rapid growth.

A repetition of the experiment in which protargol was used was made in Experiment 8.

Experiment 8. Treatment with Protargol.—11.15 a m., a *Macacus rhesus* received intraspinal inoculation of one agar slant culture, 18 hours old. 3.15 p.m., slightly ill; on perch. Lumbar puncture yielded cloudy fluid; culture positive. Films showed few leukocytes and many free meningococci. Injected 2 cc. of 0.2 per cent protargol suspension. 4.00 p.m., animal prostrate and spastic. 5.00 p.m., refused to rise. Died 5.00 a.m. next day. Survived 18 hours.

Autopsy.—No obvious visceral lesions outside the central nervous system. Turbid fluid in meninges, especially evident in sulci of cerebral convolutions. The general gray color of the cortex was about normal, but two symmetrical rectangular areas measuring 2 by 4 cm. were deeply congested and contained petechial hemorrhagic spots. They lay anterior and posterior to the fissure of Sylvius and extended to the midparietal sulcus comprising the ascending and inferior marginal convolutions. A few separate hemorrhages existed in the first temporal lobe, and the congestion and hemorrhages appeared to be confined to the gray matter. Cultures from the heart's blood and from several levels of the spinal cord and different surfaces of the brain were positive for the meningococcus. Film preparations showed many diplococci, few if any leukocytes, and little phagocytosis. Impression films prepared from the congested area of the brain contain scattered meningococci, few leukocytes, and almost no phagocytosis.

Sections from the brain show edema of and many leukocytes and red corpuscles within the meninges. The axudate contains many diplococci, both extra- and intracellular. In places the diplococci extend into the superficial layer of the cortex and slightly into the perivascular lymph spaces. These are far less numerous than in the 8 hour specimens. The affected cortex is congested and is the seat of many hemorrhages of varying size. The escape of blood is partly into the tissues and partly into the perivascular lymphatics. There is no obvious special relation between the hemorrhages and the diplococci.

A control for this monkey which received an identical quantity of an 18 hour culture was made ill, but recovered without treatment and following the spontaneous phagocytosis of the meningococci.

The differences in general disadvantageous to the protargol arose also in instances in which neither the untreated control nor the treated monkeys succumbed. In these instances cultures 24 hours old were employed. The symptoms set in more slowly than when 18 hour cultures were used and became less severe, abating wholly later.

But the monkeys given protargol suffered intensification of the symptoms almost immediately after the injection and recovered much more slowly than the untreated animals. In conformity with this fact it was found that the meningococci survived longer in the cerebrospinal fluid in the treated animals than in the control. A single experiment is given to bring out the distinction mentioned.

Experiment 9. Control.—8.50 a.m., a *Macacus rhesus* received an intraspinal inoculation of 1¾ agar slant cultures of meningococci. 2.50 p.m., slightly ill. Turbid lumbar puncture fluid gave growth. The films showed numerous leukocytes, few meningococci, and little phagocytosis. Blood cultures were negative. The symptoms progressed slowly and never became severe. 24 hours after inoculation, the animal was still ill, but improving. Lumbar puncture yielded turbid fluid, which gave negative cultures. Film preparation of the fluid showed numerous leukocytes and few meningococci all within phagocytes. Within another 24 hours the animal had recovered.

Experiment 10. Treated.—8.30 a.m., a *Macacus rhesus* received intraspinally 1½ agar slant cultures. 12.30 p.m., ill. The turbid lumbar puncture fluid gave positive cultures. The film showed many leukocytes, few meningococci, and slight phagocytosis. Injected 1 cc. of 0.2 per cent protargol suspension. 1.30 p.m., very ill. 5.00 p.m., condition unchanged; blood culture positive. Turbid lumbar puncture fluid gave positive cultures. Film showed many leukocytes, of which some contained meningococci; many extracellular meningococci also. 10.00 p.m., animal lying on bottom of cage; aroused with difficulty. 24 hours after inoculation, improving. Turbid lumbar puncture of fluid still showed intra- and extracellular diplococci. No growth was obtained in culture. At the expiration of another 24 hours, the animal had still further improved, but several days elapsed before recovery was complete.

Effects of Lysol.

The fact has already been mentioned that lysol has been employed in the treatment of epidemic meningitis with at first, as was supposed, beneficial results. Although a wider employment led very soon to its abandonment, yet the outcome of the studies of protargol suggested determining also the manner in which lysol acts. For this purpose small guinea pigs were employed. Two sets of tests were made: one in which the inoculated animals were allowed to succumb, the other in which they were chloroformed at the expiration of 6 hours, in order to ascertain just what changes were taking place in the peritoneal cavity. The lysol was employed in

1 per cent solution, which was the strength used in human cases of meningitis. The injection was made immediately following the injection of the culture, both being given into the peritoneal cavity (Table IV).

TABLE IV.

Experiment 11.   Therapeutic Test of Lysol.

Weight of guinea pig.	Quantity of culture inoculated.	Treatment.	Result.
gm			
100	1 m.l.d.	None (control).	Died in 10¾ hrs.
100	1 "	1 cc. lysol (control).	Survived.
100	1 "	0.5 cc. lysol.	Died in 7 hrs.
100	1 "	0.5 cc. lysol + 0.5 cc. antiserum.	" " 32 "
100	1 "	0.25 cc. lysol + 0.5 cc. antiserum.	" " 20 "
100	½ "	0.5 cc. lysol.	" " 12 "

Guinea Pig 1.   Control.—Peritoneal fluid clear.   0.05 cc. plated gave innumerable colonies of meningococci, as did the same quantity of heart's blood. Films of peritoneal fluid showed enormous numbers of meningococci, but no cells.   Films from the omentum showed extremely few leukocytes containing meningococci.

Guinea Pig 2.—Considerable amount of clear peritoneal fluid.   0.05 cc. plated gave innumerable colonies.   Same amount from heart's blood gave 250 colonies. Films of the peritoneal fluid showed large numbers of diplococci, well preserved but agglutinated into small clumps.   No leukocytes present.   Films of the omentum showed a considerable number of leukocytes, many containing diplococci and many extracellular diplococci agglutinated.

Guinea Pig 3.—Very small amount of turbid peritoneal fluid.   0.05 cc. plated gave innumerable colonies.   Same quantity of heart's blood gave 200 colonies of meningococci.   Films of peritoneal fluid showed numerous leukocytes filled with diplococci, and large numbers of diplococci arranged in small clumps remaining extracellular.

Guinea Pig 4.—Small amount of turbid peritoneal fluid.   0.05 cc. plated gave 1,000 colonies.   Same quantity of heart's blood gave no colonies.   Films of peritoneal fluid showed numerous leukocytes, some staining badly, many containing diplococci, and a considerable number of small clumps of extracellular diplococci, some partly disintegrated.   Films of the omentum showed more phagocytosis and a smaller number of extracellular diplococci.

Guinea Pig 5.—A considerable amount of turbid peritoneal fluid.   0.05 cc. plated gave innumerable colonies, as did the heart's blood.   Films of the peri-

toneal fluid showed many leukocytes and a small amount of phagocytosis, besides extracellular clumps of diplococci. Films of the omentum showed more phagocytosis than the peritoneal fluid and a larger number of leukocytes, besides extracellular diplococci.

Guinea Pig 6.—A considerable amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies, as did the heart's blood. Films of the peritoneal fluid showed numerous leukocytes, a small amount of phagocytosis, and numerous clumped extracellular diplococci. Films of the omentum showed numerous leukocytes, slight phagocytosis, and extracellular diplococci occurring in clumps.

Mechanism of the Action of Lysol.

The mode of the action of lysol was studied by inoculating three young guinea pigs with one minimal lethal dose of culture and treating them immediately with 1 per cent lysol or 1 per cent lysol plus antiserum, as indicated in Table V.

TABLE V.

Experiment 12.

Weight of guinea pig.	Quantity of culture inoculated.	Treatment immediately after inoculation.	Condition after 6 hrs.
gm.			
100	1 m.l.d.	0.5 cc. lysol + 0.5 cc. water.	Dead.
100	1 "	0.25 " " + 0.75 " "	Very sick.
100	1 "	0.25 " " + 0.25 " " + 0.5 cc. antiserum.	Prostrate.

The two guinea pigs which still survived were chloroformed and autopsies performed at once.

Guinea Pig 1.—Peritoneal fluid clear. 0.05 cc. plated gave innumerable colonies of meningococci. Same quantity of heart's blood gave 100 colonies. Films of the peritoneal fluid showed many meningococci and few leukocytes, there being almost no phagocytosis, while the leukocytes stained feebly. The meningococci were chiefly aggregated into clumps of 10 or 20 individuals. They stained as a rule diffusely and not sharply. The omentum showed many more leukocytes, almost all containing meningococci singly and in small clumps. The phagocytes stained feebly.

Guinea Pig 2.—Peritoneal fluid clear. 0.05 cc. gave innumerable colonies of meningococci. Same quantity of heart's blood gave about 100 colonies. Films of peritoneal fluid and omentum showed a condition similar to No. 1.

Guinea Pig 3.—Peritoneal fluid turbid, gave 150 colonies of meningococci and the same quantity of heart's blood gave three colonies. Films from the peritoneal cavity showed no diplococci whatever, either free or in leukocytes, of which a good number in excellent preservation were present. Films from the omentum showed large numbers of well preserved leukocytes, of which a few contained meningococci, either sharply stained or disintegrating; extracellular diplococci were practically absent.

It may in brief be stated that the experiments with guinea pigs indicate that lysol is antileukotactic to an even greater degree than is protargol. Moreover, the antiserum is capable in this case of overcoming to even a further extent the negative chemotactic and antiphagocytic action of the chemical. Obviously the combined toxic power of the chemical and the disintegrating effect upon the meningococci, notwithstanding the action of the serum, bring about a fatal issue.

DISCUSSION.

The results of the tests with protargol and with lysol are consistent. The chemicals have shown themselves not to be curative, but rather to be injurious in experimental meningococcic infection in guinea pigs and monkeys. Far from exhibiting power to convert a fatal into a non-fatal issue, they have shown rather a reverse tendency. Even when, as in certain tests on monkeys, recovery occurred after employing protargol, the symptoms of the treated animals were intensified as compared with the untreated control animals.

The explanation of the unfavorable effect of the chemicals is supplied by the study of the processes occurring in the peritoneum of guinea pigs and the subarachnoid space of monkeys. The key to recovery from infection with the meningococcus is furnished by the phenomenon of phagocytosis. Whatever means promote phagocytosis under conditions in which the leukocytes remain potent, facilitate recovery; whatever means lead to the reverse effect interfere with recovery. The chemicals have been shown to be antileukotactic, antiphagocytic, and indeed to be cell poisons of considerable power. Hence when they are brought into relation with the seat of infection, they prevent emigration of leukocytes on an adequate scale, and they reduce phagocytosis by such leukocytes as have entered the serous cavities.

Moreover, they also injure and bring about degeneration of the leukocytes themselves.

The resultant of this set of injurious activities is to open the way for a free invasion of the blood by the meningococci and for almost unrestrained multiplication in the serous cavities.

The chemicals do exert a direct bactericidal action upon such meningococci as come under their influence under conditions of suitable concentration, but not all the meningococci are thus destroyed. Those which survive multiply almost without hindrance; while the absence of detoxicating power on the part of the chemical permits the disintegration products of the destroyed meningococci to exert their poisonous effect upon the animal organism.

The manner of action of the chemicals is precisely the reverse of the antiserum. The latter acts leukotactically and brings into the infected serous cavities a far larger number of leukocytes than would otherwise appear there in the same period of time. Moreover, by preparing the meningococci for phagocytosis by supplying opsonin and by facilitating that process by agglutinating the diplococci, the serum greatly promotes the englobing of the microorganisms. Finally, through the possession of antitoxic power it neutralizes whatever endotoxin may be liberated by the disintegrating diplococci.

It is, therefore, not remarkable to find, as the experiments have shown, that the antiserum is capable of overcoming part of the defects of the chemicals. Because of its leukotactic and phagocytic properties, the antiserum removes some of the damage which the chemicals produce on account of their antagonism to those essential phenomena. But combinations of the chemical and the antiserum have proven less effective in combating experimental meningococcic infection than the antiserum alone, from which the deduction may be made that whatever benefit may attach to effective direct bactericidal action by the chemicals, irrespective of the type of meningococcus causing the infection, is lost because of the dangers arising from their antiphagocytic effects and lack of detoxicating properties.

Doubtless the introduction of the chemicals into the subarachnoid space in man has been made without the uniformly serious consequences observed in our experiments on animals. The reason for this

discrepancy is perhaps obvious. The relative dosage, considering comparative size and weight, was far less in man than in the animals. Hence it may be assumed that the injurious effects, if any were produced, were masked. To assume, on the other hand, that because no evidence of untoward action was detected in the treatment with protargol, and five out of eight cases of epidemic meningitis treated with that chemical recovered, the drug has curative properties, is to disregard previous experience with simple lumbar puncture, with lysol, and with other methods of treatment which for a time and because of the variable severity of cases of epidemic meningitis seemed to offer encouragement. It is not probable that such active protoplasmic poisons as protargol and lysol can be employed with impunity for direct introduction into the closed cavity of serous membranes, the seat of the meningococcic infection.

SUMMARY.

Claims of efficiency have been made at two widely separated periods for the chemical treatment of epidemic meningitis, in the first instance for lysol and in the second for protargol. The use of lysol was long since abandoned; the recommendation for protargol is based on a single series of cases, small in number. Because of the variable severity of epidemics of meningitis, small reliance can be placed on results of treatment limited in extent to small numbers of cases and to one locality. A more uniform and accurate measure of the value of a method of treatment is provided by animals infected experimentally with pathogenic cultures of meningococci.

Young guinea pigs respond in a definite manner to intraperitoneal inoculation of virulent meningococci. Neither protargol nor lysol proved to have any curative action on the experimental infection thus produced in these animals.

Monkeys respond in a characteristic manner to the inoculation of virulent cultures into the subarachnoid space. Protargol displayed no curative action on the experimental infection thus produced in these animals.

On the contrary, both lysol and protargol exert antileukotactic and antiphagocytic effects, and are also potent protoplasmic poisons,

and the leukocytes with which they come in contact are injured and made to degenerate. According to the extent to which these harmful properties are exerted, the chemicals promote the advance rather than restrain the progress of meningococcic infection.

Recovery from meningococcic infection in man and animals is accomplished chiefly through the process of phagocytosis. The specific antiserum acts curatively by increasing the emigration of leukocytes, by promoting phagocytosis directly, and by agglutinating the meningococci, and also by neutralizing endotoxin. Any means which interfere with and reduce these essential processes retard or prevent recovery. Both lysol and protargol interfere with and diminish the emigration of leukocytes and the phagocytosis of meningococci, and neither possesses antitoxic power.

The mixture of antiserum with lysol and with protargol reduces to a certain extent the antileukotactic and antiphagocytic effect of the chemicals; but this action is insufficient to set aside wholly the injurious effects which they produce.

It follows, therefore, that whatever theoretical advantages might accrue from a bactericidal activity exerted by these chemicals independently of the type of meningococcus causing epidemic meningitis, is more than offset by the harmful effects which they cause.

Hence specific antiserum seems to provide the logical therapeutic agent with which to combat epidemic meningitis, since it is itself innocuous and promotes those processes essential to recovery from the disease. The problem up to the present has been that of producing an antiserum which represents the several types of the meningococcus, and this problem is now in a fair way to being solved.[1]

THE DISAPPEARANCE OF DEXTROSE FROM THE BLOOD AFTER INTRAVENOUS INJECTION.

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HISTORICAL.

Claude Bernard[1] was the first to study the elimination of sugars after intravenous administration. He, as well as others,[2] demonstrated that dextrose, lactose, or saccharose, when thus injected in large quantity, is eliminated in the urine. Although working only qualitatively, he[3] realized that dextrose was better assimilated under these circumstances than saccharose or lactose. The time relations were worked out, particularly by von Becker, who found after injecting 1.5 gm. of dextrose into rabbits that sugar elimination by the kidneys began promptly and continued for 5 or 6 hours. Limpert and Falck[4] did the first quantitative work. Dogs of 5 or 6 kilos when given 5 to 7 gm. of dextrose intravenously excreted only traces in the urine. Larger amounts led to a slightly increased excretion. Thus it was shown early that after intravenous injection fairly large amounts of dextrose can be retained. The proportion excreted in the urine, according to von Brasol[5] and others[6] bears no constant relation to the injected dextrose; nor can the duration of the glycosuria be predicted. Von Brasol also found that the degree and duration of glycosuria were not always the same for

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[1] Bernard, C., Dissertation, Neue Funktion der Leber als zuckerbereitendes Organ des Menschen und der Thiere, Würzburg, 1853.

[2] Kersting, *Jour. f. prakt. Chem.*, 1844, xxxiii, 58. Baumert, M., *Jour. f. prakt. Chem.*, 1851, liv, 357. Uhle, Inaugural Dissertation, Leipzig, 1852. von Becker, F. J., *Ztschr. f. wissensch. Zool.*, 1854, v, 123. All quoted by Limpert, L., and Falck, C. P., *Virchows Arch. f. path. Anat.*, 1856, ix, 56.

[3] Bernard, C., *Compt. rend. Acad.*, 1846, xxii, 536.

[4] Limpert, L., and Falck, C. P., *Virchows Arch. f. path. Anat.*, 1856, ix, 56.

[5] von Brasol, L., *Arch. f. Physiol.*, 1884, 211.

[6] Pavy, F. W., *Jour. Physiol.*, 1899, xxiv, 479. Weyert, F., *Arch. f. Physiol.*, 1891, 187. Lilienfeld, C., *Ztschr. f. diätet. u. physik. Therap.*, 1898–99, ii, 209. Wilenko, G. G., *Arch. f. exper. Path. u. Pharm.*, 1911, lxvi, 143. Bang, I., Der Blutzucker, Wiesbaden, 1913, 76.

a given animal.   Gilbert and Carnot,[7] however, maintained that within certain limits the proportion of sugar eliminated is constant for each animal, and Blumenthal[8] found that the "assimilation limit;" *i.e.*, the amount which can be injected intravenously without causing glycosuria, is practically invariable for each individual.   The velocity of injection plays a part, for Doyon and Dufourt[9] found that more is excreted after rapid than after slow injection.   According to them, age, state of nutrition, ligation of the bile duct, and administration of alcohol are apparently without influence.   Bang[10] has shown that the glycosuria and glycemia do not run parallel.   The concentration of the injected sugar probably plays a part, for Wilenko[11] states that concentrated dextrose solutions (40 per cent) produce the same changes in renal permeability as concentrated salt solutions; that is, first an increased and then a decreased permeability for sugar.

During the injection of dextrose the blood sugar rises and afterwards falls, until it finally assumes a normal or subnormal figure.   Von Brasol[5] injected from 0.9 to 5.3 gm. per kilo of body weight in 4 to 6 minutes.   Analysis indicated that in the first 2 minutes following the injection some of the sugar had already left the blood; but no direct ratio existed between the amount injected and the blood sugar percentage.   After 2 hours the sugar content of the blood was usually normal; with smaller doses (0.9 and 2.3 gm. per kilo) this level was sometimes reached in 1 hour.   Butte[12] injected 3 to 10 gm. of dextrose per kilo intravenously and noted the rapid fall of blood sugar to or nearly to the normal level.   With 4 gm. per kilo for example, the percentage of sugar in the blood 1½ hours after the injection was 0.353 per cent, and 2 hours after, 0.220 per cent.   In no case did he find a subnormal figure as did Grèhant[13] who found 0.036 per cent blood dextrose 2 hours after an injection of 6 gm. of dextrose per kilo.   Pavy[14] found in rabbits that the blood sugar fell rapidly after an intravenous injection; for example, immediately after 4 gm. per kilo had been injected, the blood contained 1.4 per cent sugar; 5 minutes after, 1 per cent; and 15 minutes after, 0.8 per cent. Lépine[15] found that after the injection of 1 gm. of dextrose per kilo slowly into dogs, the blood sugar fell in the course of an hour below 0.1 per cent.   Bang[16] injected 1 to 2 gm. per kilo intravenously and found that the maximum blood sugar was reached 2 to 5 minutes after the injection and that the high percentage was maintained for about 30 minutes, after which a rapid fall to normal or below

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[7] Gilbert and Carnot, *Compt. rend· Soc. de biol.*, 1898, v, 330.

[8] Blumenthal, F., *Beitr. z. chem. Phys. u. Path.*, 1905, vi, 329.

[9] Doyon, M., and Dufourt, E., *Jour. de physiol. et de path. gén.*, 1901, iii, 703.

[10] Bang, I., Der Blutzucker, Wiesbaden, 1913, 77.

[11] Wilenko, G. G., *Arch. f. exper. Path. u. Pharm.*, 1911, lxvi, 143.

[12] Butte, L., *Compt. rend. Soc. de biol.*, 1896, iii, 274.

[13] Grèhant, quoted by Butte, L., *Compt. rend. Soc. de biol.*, 1896, iii, 274.

[14] Pavy, F. W., *Jour. Physiol.*, 1899, xxiv, 479.

[15] Lépine, R., Le diabète sucré, Paris, 1909, 200.

[16] Bang, I., Der Blutzucker, Wiesbaden, 1913, 74.

took place. Using animals with ligated ureters, Weyert[17] found that after the injection of 4.4 to 5.8 gm. of dextrose per kilo, the blood sugar was down to nearly normal limits in 3 hours, and Harley[18] found that with a dosage of 10 gm. per kilo, the blood sugar fell to normal in from 3 to 6 hours.

Weyert[17] records that the sugar concentration of the lymph runs close to that of the blood. He also found traces of sugar in the cerebrospinal fluid and in the vitreous humor of the eye. None can be found in the saliva except after very large doses,[9, 17, 19] nor is an appreciable amount secreted by the intestine[9, 20, 21] or by the wall of the urinary bladder.[22]

The possibility of the conversion of injected dextrose into glycogen must, of course, be considered. Voit[23] injected subcutaneously 50 gm. of dextrose into three rabbits and found in the liver only 1.4, 2.2, and 7.0 gm. of glycogen, respectively, showing that sugar is not as readily converted into glycogen by the liver when injected parenterally as when fed. Harley[18] after injecting 10 gm. of dextrose per kilo intravenously into dogs with the ureters tied, found some slight evidence of glycogen formation. Freund and Popper[24] analyzed a lobe of liver before injecting dextrose intravenously and the rest of the liver afterwards, and found a small increase in liver glycogen, which was greater if the animals had previously been starved for a short time. With starved dogs 4 gm. per kilo gave no increase in glycogen, but 7 to 11 gm. per kilo resulted in the formation of 1.3 to 7 gm. of glycogen.

Inasmuch as the injected sugar is accounted for only in small part by glycogen and by the various secretions, the blood at the same time rapidly assuming its normal content of sugar, the question naturally arises: What is the fate of the rest of the sugar? In seeking to answer this question, various facts have been brought out. Von Brasol[5] injected 12 to 18 gm. of dextrose per kilo intravenously into rabbits in 30 to 45 minutes and then analyzed the blood, the urine, and mixed samples of muscle, kidney, and liver. Estimating the relation of blood to body weight even as high as 12 per cent and even assuming that all the tissues, including bones, hair, etc., contained the same proportion of dextrose as the tissues analyzed, he calculated that from 17.5 to 28.7 per cent of the sugar administered, was still to be accounted for. Von Brasol did not determine glycogen or other substances; in fact he suggests that the missing fraction has been converted into glycogen, or lactic acid or some other substance. Butte[12] made muscle analyses after injecting intravenously 4 gm. of dextrose per kilo into a dog. Assuming

[17] Weyert, F., *Arch. f. Physiol.*, 1891, 187.

[18] Harley, V., *Arch. f. Physiol.*, 1893, Supplement, 46.

[19] Jappelli, A., *Ztschr. f. Biol.*, 1908, li, 435.

[20] Bang, I., Der Blutzucker, Wiesbaden, 1913, 80.

[21] Kleiner, I. S., *Jour. Exper. Med.*, 1911, xiv, 274.

[22] Kleiner, I. S., *Jour. Exper. Med.*, 1913, xviii, 310.

[23] Voit, C., *Ztschr. f. Biol.*, 1891, xxviii, 245.

[24] Freund, E., and Popper, H., *Biochem. Ztschr.*, 1912, xli, 56.

that the muscle contained no sugar before the injection, he noted a rise to 0.42 per cent half an hour after the injection and then a progressive fall in the percentage of dextrose. This demonstrates, according to Butte, that one part of the injected sugar is transformed in certain organs, although he does not mention the likelihood of bacterial action in a dead animal. In two experiments on rabbits Bang[20] analyzed the liver, skin, blood and lymph, kidneys and urine, muscles, intestines, and bones for free dextrose after injecting 4 gm. intravenously into each animal. The total amount recovered was 47.9 and 75.5 per cent, respectively. But, according to Bang, even these figures are too high because (a) preformed sugar was not determined, (b) the tissues were not blood-free and hence blood sugar was counted twice, and (c) all reduction was not necessarily due to sugar.

The rapid transformation of dextrose into simpler substances, particularly lactic acid, has been suggested by many investigators. Apparently the only one who actually tested for lactic acid after intravenous injections of dextrose was Harley.[18] He found a higher percentage of lactic acid in the blood after dextrose injections into animals with the ureters tied, and also an increase in this substance in the liver and muscle. He also obtained qualitative tests for ethyl alcohol and acetone in the blood. Although some workers have brought forth evidence in favor of a protein origin for lactic acid, there seems to be no doubt that it can be formed from sugar. For example, Embden and his collaborators[25] by perfusing livers with blood found that the liver could form lactic acid from glycogen in the liver or dextrose in the blood. More recently Levene and Meyer[26] have observed a direct conversion of sugar into lactic acid by leukocytes.

An increase in the respiratory quotient after intravenous dextrose injections has been observed by Harley,[27] Verzár and Fejér,[28] and others.

The following points appear, therefore, to be established: (1) The intravenous injection of large quantities of dextrose is followed by an increase in the percentage of sugar in the blood. (2) The high content of sugar in the blood falls rapidly after the end of the injection and assumes the normal value in a comparatively short time, even when the ureters are tied. (3) The kidneys eliminate a considerable, but variable, fraction of the injected sugar. (4) No sugar, or mere traces are to be found in the cerebrospinal fluid, saliva, intestinal secretions, or in the secretion of the mucosa of the urinary bladder. (5) An increase of glycogen in the liver occurs with very large doses, but it accounts for only a small fraction of the sugar injected. (6) Tissue analyses, although few in number, show that some of the sugar is to be found unaltered in various organs. (7) Some of the sugar is oxidized, or at least the respiratory quotient is increased.

[25] Embden, G., *Centralbl. f. Physiol.*, 1904–05, xviii, 832. Embden, G., and Kraus, F., *Biochem. Ztschr.*, 1912, xlv, 1.

[26] Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1912, xi, 361.

[27] Harley, V., *Jour. Physiol.*, 1894, xv, 139.

[28] Verzár, F., and von Fejér, A., *Biochem. Ztschr.*, 1913, liii, 146.

The chief purpose of the present work was to study the disappearance of sugar from the blood under various conditions. In order to obtain a basis of comparison a series of experiments was first carried out in which dextrose was injected into normal animals.

EXPERIMENTAL PART.[29]

The experiments were performed on dogs. Ether was administered by intratracheal insufflation, and cannulas were inserted in the left jugular vein, the right carotid artery, and the neck of the urinary bladder. In a few of the later experiments only morphine and cocaine were employed as anesthetics. In the experiments in which the kidneys were ligated, they were reached through the lumbar region. At the completion of all operative work, the animal was given morphine, usually 0.01 gm. per kilo, subcutaneously, and then the insufflation and ether anesthesia were discontinued. After $1\frac{1}{2}$ to $2\frac{1}{4}$ hours a sample of blood was taken from the artery, and a warm solution of 20 per cent dextrose was injected slowly into the jugular vein. The rate of injection was about 2.5 to 3 cc. per minute and the dosage was 4 gm. per kilo of body weight. At the middle and end of the injection and at intervals thereafter, samples of blood (from 10 to 30 gm.) were taken for analysis. Urine elimination was divided into periods corresponding to the blood samples taken. During the experiment the animal was kept on an electric thermal pad and at the end of the experiment it was chloroformed.

The blood was analyzed for dextrose by removing the protein by Reid's method,[30] and the reduction was determined by means of a Pavy[31] solution. The urine

[29] A preliminary report of this work was published by Kleiner, I. S., and Meltzer, S. J., *Am. Jour. Physiol.*, 1914, xxxiii, p. xvii.

[30] Reid, E. W. *Jour. Physiol.*, 1896, xx, 316. Vosburgh, C. H., and Richards, A. N., *Am. Jou,r. Physiol.*, 1903, ix, 35. Macleod, J. J. R., *Jour. Biol. Chem.*, 1908–09, v, 443.

[31] The reagent used was modified from time to time. Vernon (*Jour. Physiol.* 1902, xxviii, 156) suggested the use of twice as much Rochelle salt, potassium hydroxide, and ammonium hydroxide as Pavy originally employed. As this results in the formation of a heavy crystalline deposit in the reagent on standing, we have decreased the amount of Rochelle salt and potassium hydroxide. The resulting solution, which deposits very little precipitate, gives good results. The composition of the solution, as we now prepare it, is:

$CuSO_4$	4.158 gm.	
Rochelle salt	14	"
KOH	17	"
NH_4OH (sp. gr. 0.88)	600 cc.	
H_2O to	1,000	"

10 cc. of this solution corresponds to 0.005 gm. dextrose, but one should determine the exact equivalent every time the reagent is made up, and this should be checked at intervals.

was also analyzed by the Pavy method. Although this method is not as accurate as some of the other sugar methods, its rapidity, and the fact that a number of determinations can be made from a small quantity of a given solution (as each test requires only 5 mg. of dextrose), led us to use it in this investigation. In some experiments, however, the Bertrand method was used to determine the reducing power of the blood after removal of the proteins, and in a few of the experiments we have used the blood sugar method of Lewis and Benedict as modified by Myers and Bailey,[32] which we have recently adopted for use in this laboratory. With this method only 2 cc. of blood is required.

Normal Animals.

The first dextrose injections were given to seven normal animals. The following is a typical protocol.

Experiment LD 5.—White bull terrier, female; weight 6.5 kilos.

11.00. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. Cannulas now inserted in the left jugular vein, left carotid artery, and in the neck of the bladder.

11.51. Urine obtained from bladder; 1.69 per cent dextrose.

11.56. Ether discontinued.

12.02. 6.5 cc. 1 per cent morphine sulphate injected subcutaneously.

12.13. Insufflation discontinued.

2.07. Blood taken, 22.3 gm.; 0.28 per cent dextrose.

2.09. Urine, 77 cc. + (about 5 cc. lost), 8.84 per cent = 6.81 gm. dextrose.

2.10. Injection of warm 20 per cent dextrose solution into jugular vein started.

2.34. Blood taken, 9.5 gm., 0.68 per cent (67 cc. of dextrose have been injected).

2.36. Urine, 40 cc., 6.84 per cent = 2.74 gm. (70 cc. of dextrose have been injected).

2.56. Dextrose injection ends. Total amount injected 130 cc., or 26 gm. in 46 minutes.

2.57. Blood taken, 10.0 gm., 0.93 per cent.

2.58. Urine, 81 cc., 5.50 per cent = 4.46 gm.

3.27. Blood taken, 16.6 gm., 0.40 per cent.

3.28. Urine, 51 cc., 7.32 per cent = 3.73 gm.

3.57. Blood taken, 17.9 gm., 0.30 per cent.

3.58. Urine, 11.5 cc., 10.6 per cent = 1.22 gm.

4.27. Blood taken, 21.3 gm., 0.28 per cent.

4.29. Urine, 3.2 cc., 8.59 per cent = 0.28 gm.

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[32] Lewis, R. C., and Benedict, S. R., *Jour. Biol. Chem.*, 1915, xx, 61. Myers, V. C., and Bailey, C. V., *Post-Graduate*, 1915, xxx, 31; *Jour. Biol. Chem.*, 1916, xxiv, 147.

Summary.—1½ hours after the end of the dextrose injection the blood sugar fell to its original level of 0.28 per cent.

	$gm.$
Dextrose injected..	26.0
Excess dextrose (above original value) in circulating blood at end of experiment..	0.00
Dextrose contained in blood samples taken for analysis.........	0.13
Dextrose in urine..	12.43
Dextrose accounted for....................................	12.56
(or 48.5 per cent of the amount injected)	
Dextrose not accounted for................................	13.44
(or 51.5 per cent of the amount injected)	

The results of the seven experiments are given in Table I.

Considering first the glycemia, it is seen that in five out of seven experiments, the original blood sugar was high. This was to be expected, as the animals had been subjected to being tied down, to anesthesia (ether and morphine), and to operative procedure, all of which are known to raise the blood sugar.[33] An initial rapid rise in the blood sugar during the injection is indicated in each case by the figures for the beginning and middle of the injection. Then a more gradual rise occurred until the end of the injection, after which the percentage of blood sugar fell rapidly for half an hour and then more slowly. At the end of 1½ hours the percentage of sugar in the blood fell to its original level in three experiments (Experiments 3, 5, and 46) and nearly to its original level in two others (Experiments 6 and 7). In the two remaining experiments (4 and 47) the last figures were twice as great as the first. However, in one of these two (Experiment 47) the last percentage found was, nevertheless, a low figure when compared with the rest of the series. In other words, in only one of the seven experiments did the blood sugar fail to fall to what might be termed a low figure for this series.

In Experiments LD 4, 5, and 6, and LP 46, the urine secreted during the injection contained more sugar than that secreted during the 90 minute after-period. This was due to a greater volume of urine being secreted at first rather than a higher percentage of sugar.

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[33] For a discussion of these points see Shaffer, P. A., *Jour. Biol. Chem.*, 1914, xix, 297. Loewy, A., and Rosenberg, S., *Biochem. Ztschr.*, 1913, lvi, 114. Hirsch. E., and Reinbach, H., *Ztschr. f. physiol. Chem.*, 1914, xci, 292.

TABLE I.

Intravenous Injection of Dextrose into Normal Dogs.

No. of experiment	Dextrose injected (4 gm. per kilo) gm.	cc.	Blood sugar before injection per cent	Middle of injection Blood sugar per cent	Middle Urine Volume cc.	Middle Urine Dextrose per cent	Middle Urine Dextrose gm.	End of injection Blood sugar per cent	End Urine Volume cc.	End Urine Dextrose per cent	End Urine Dextrose gm.	½ hr. after injection Blood sugar per cent	½ hr. Urine Volume cc.	½ hr. Urine Dextrose per cent	½ hr. Urine Dextrose gm.	1 hr. after injection Blood sugar per cent	1 hr. Urine Volume cc.	1 hr. Urine Dextrose per cent	1 hr. Urine Dextrose gm.	1¼ hrs. after injection Blood sugar per cent	1¼ hr. Urine Volume cc.	1¼ hr. Urine Dextrose per cent	1¼ hr. Urine Dextrose gm.	Total volume of urine cc.	Total amount of sugar in urine gm.	Per cent of total amount injected	Sugar not accounted for by blood and urine. Per cent of total amount injected
LD3...	29.6	148	0.23	0.85	0			0.98	0			0.63	10.5	3.68	0.39	0.33	1.0	5.5	0.05	0.24	2.7	3.7	0.10	15	0.54	1.8	97.0
LD4...	26.8	134	0.20	0.71	35	6.62	2.32	0.80	81	7.31	5.92	0.45	637	.70	4.86	0.47	8.3	9.38	0.78	0.41	9.9	9.06	0.90	197	14.78	55.1	39.9
LD5...	26.0	130	0.28	0.68	40	6.84	2.74	0.93	81	5.50	4.46	0.40	51	7.32	3.73	0.30	11.5	10.61	1.22	0.28	3.2	8.59	0.28	187	12.43	47.8	51.5
LD6...	26.0	130	0.19	0.56	32	9.80	3.14	0.72	61	9.80	5.98	0.40	49	9.22	4.52	0.28	13.5	10.91	1.47	0.25	11.5	11.54	1.33	167	16.44	63.2	34.2
LD7...	30.0	150	0.11	0.49				0.50				0.25	121	8.68	10.50	0.18				0.15	50	8.06	4.03	171	14.53	48.4	50.4
LP46*..	34.0	170	0.18					0.70	209	7.42	15.51									0.20	112	10.53	11.79	321	27.30	80.3	19.4
LP47*..	35.6	178	0.11					0.76	112	10.10	11.31									0.25	92	13.32	12.25	204	23.56	66.2	31.4
Average			0.19	0.16†				0.77				0.43†				0.33†				0.25						60.2‡ 51.8§	37.8‡ 46.3§

* Only morphine anesthesia.   † Average of five.   ‡ Average of six.   § Average of seven.

266

Indeed, the urine of the after-period was usually of a higher concentration, and in Experiment LP 47 this was sufficient to cause a greater excretion of sugar in the after-period than in the injection period.

The small flow of urine in Experiment LD 3 was probably due to over-etherization. Excluding this experiment, the average amount found in the urine was 60.2 per cent of the sugar injected. If we now estimate the excess sugar still circulating in the blood (calculating the blood equal to 7 per cent of the body weight) and add this blood sugar to the urinary sugar, we have an average of 62.2 per cent of the injected amount, leaving 37.8 per cent not accounted for. If we include Experiment LD 3, this average is raised to 46.3 per cent not accounted for.

It thus appears from our own experiments on normal animals that approximately 60.2 per cent of the sugar was excreted by the kidneys, while of the remaining 39.8 per cent only an insignificant fraction (2 per cent on an average) remained in the circulation.

Nephrectomized Animals.

In one of our experiments on normal animals (Experiment LD 3) there occurred, as mentioned above, an almost complete suppression of the kidney function during and after the intravenous infusion of dextrose. Nevertheless, the blood sugar returned to its original value as quickly as in the other experiments. We now performed some experiments upon animals after ligating or removing the kidneys. The results of five experiments are summarized in Table II. The following is a typical protocol.

Experiment LD 33.—Fox-terrier, male; weight 6.75 kilos.

10.18. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. The kidneys were now exposed and ligated and cannulas inserted in left jugular vein and right carotid artery.

11.15. Ether discontinued.

11.26. Lid reflex present.

11.27. Blood taken, 11.6 gm., 0.28 per cent.

11.31. Insufflation discontinued.

11.33. 1 cc. 1 per cent morphine sulphate injected intramuscularly.

12.27. Blood taken, 11.35 gm., 0.27 per cent.

12.28. Injection of warm 20 per cent dextrose solution into jugular vein started.

1.22. Dextrose injection ended. Total amount introduced, 135 cc. or 27 gm. in 54 minutes.

1.23. Blood taken, 11.9 gm., 1.08 per cent.

1.29. 0.5 cc. 1 per cent morphine sulphate injected intramuscularly.

1.54. Blood taken, 11.5 gm., 0.69 per cent.

2.24. Blood taken, 14.9 gm., 0.40 per cent.

2.54. Blood taken, 15.95 gm., 0.32 per cent.

Summary.—1½ hours after the end of the injection the blood sugar fell to 0.32 per cent; *i.e.*, only 0.05 above its original level of 0.27 per cent.

	gm.
Dextrose injected..	27.0
Excess dextrose (above original value) in circulating blood at end of experiment................................... about	0.27
Dextrose contained in blood samples taken for analysis .... about	0.17
Total dextrose thus accounted for............................	0.44
(or 1.7 per cent of the amount injected)	
Dextrose not accounted for................................	26.56
(or 98.3 per cent of the amount injected)	

TABLE II.

Intravenous Injection of Dextrose into Nephrectomized Dogs.

No. of experiment.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.		Blood sugar at end of injection.	Blood sugar ½ hr. after injection.	Blood sugar 1 hr. after injection.	Blood sugar 1½ hrs. after injection.	Blood sugar 2 hrs. after injection.	Sugar not accounted for. Per cent of total amount injected.
			Before morphine.	After morphine.						
	gm.	cc.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
LD 32	11.0	55		0.47	1.09	0.93	0.70		0.45	100
LD 33	27.0	135	0.28	0.27	1.08	0.69	0.40	0.32		98.3
LD 34	42.0	210	0.30	0.35	1.0		0.55	0.46	0.29	99.6
LP 48*	43.4	217		0.10	0.62			0.13		99.5
LP 49*	37.0	185		0.28	1.03			0.27		100
Average				0.29	0.96			0.295		99.5

*In these two experiments the kidneys were exposed and prepared for ligation under ether anesthesia, but they were not actually ligated until the effects of the ether had worn off.

In these animals the blood sugar rises to a higher level, but this is not as great as might be expected. In the normal dogs the average figures were 0.19 before injection and 0.77 at the end, a rise of 0.58 per cent; in the nephrectomized animals the corresponding figures are 0.29 and 0.96, a rise of 0.67 per cent; hence there is a difference between the two series of only 0.09 per cent. If we now estimate from this figure how much more sugar there is circulating in the blood in this series, we find that it amounts to less than 1 gm. Therefore during the injection the organism without the kidney has been enabled to rid its blood of nearly as much sugar as if it had its renal function intact. After the discontinuation of the injection the fall in the glycemia is most rapid during the first hour and then becomes slower. In Experiments LD 33, LD 34, LP 48, and LP 49 the blood sugar is either at its original level or only slightly higher at the end of 1½ hours, and in Experiments LD 32 and 34 the figures for 2 hours show that by this time the blood sugar has fallen to or below this value. In Experiment 32 no sample was taken at the end of 1½ hours, but the average for the other four experiments for this time was 0.30 per cent, while the average before injection was 0.25 per cent in the same four.

It is thus evident that after an intravenous injection of dextrose the blood sugar does not rise much higher in nephrectomized animals than in normal animals, and after injection gradually falls to or nearly to its original level. This indicates, of course, that practically all the injected sugar has left the blood stream; and that therefore the body is able to dispose of large amounts of injected dextrose even without the assistance of the kidneys. Furthermore, the fact that this was accomplished in the same length of time as in the normal series, the dosage and other experimental conditions remaining the same, shows that the presence or absence of the kidney has very little influence on the rate of disappearance of dextrose from the blood.

The obvious explanation which offers itself at first thought is that the greater part of the injected sugar is rapidly converted into glycogen by the liver. We have performed some experiments which were designed to exclude this possibility. Dextrose was injected into dogs in which there was practically no circulation posterior to the diaphragm. Animals can easily be prepared in this manner since, with

the aid of intratracheal insufflation, the thorax can be opened and the aorta and vena cava inferior tied near the diaphragm with great facility.

Animals with a Circulation only Anterior to the Diaphragm.

The animals were prepared as follows: An opening was made in the left side of the thorax (under intratracheal insufflation anesthesia) and the aorta and vena cava were ligated as near the diaphragm as possible. The aorta was ligated first, and before ligating the vena cava, pressure was applied to the abdomen in order to increase the amount of blood in the thorax. In some cases Ringer's solution or saline, in others adrenalin, or both, were injected to maintain the blood pressure. These animals, then, had no abdominal circulation; the liver could store no glycogen; the kidneys, pancreas, and adrenals, could not function. Into such animals, we injected dextrose as before and estimated the glycemia at intervals. Some experiments were complicated by such variations as removal of the thyroids, tying the thoracic duct, etc. These additional factors exerted no appreciable influence on the results, and we shall not deal with them in particular. Some of the experiments are summarized in Table III.

A typical experiment of this series is the following:

Experiment LD 30.—Dog; weight 7.5 kilos.
1.40. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. Cannulas now inserted in left jugular vein and right carotid artery. Thoracic duct exposed. Opening made in left side of thorax and aorta tied near diaphragm. After pressure on the abdomen the vena cava inferior was tied near diaphragm.
2.43. Wound in thorax closed. ·
2.44. Ether discontinued.
2.46. Thoracic duct tied.
2.47. Blood taken, 10.9 gm., 0.07 per cent.
2.48–2.50. 40 cc. warm sterile Ringer's solution injected in left jugular vein.
3.04–3.05. 20 cc. Ringer's solution injected.
3.18. 10 cc. Ringer's solution injected.
3.23. Pulse poor. Dog restless. 20 cc. Ringer's solution injected.
3.31–3.32. 20 cc. Ringer's solution injected. (Total quantity Ringer's solution injected, 110 cc. during 44 minutes).
3.40. 1 cc. 1 per cent morphine injected intramuscularly in left fore-leg.
3.49. Blood taken, 10.8 gm., 0.05 per cent.
3.50. Injection of warm 20 per cent dextrose in left jugular vein started.
4.30. Dextrose injection ended. Total amount injected, 150 cc. 20 per cent dextrose or 30 gm., in 40 minutes.

4.32. Blood taken, 14.7 gm., 1.65 per cent.
4.48. Pulse good.
5.27. Blood taken, 22.6 gm., 1.16 per cent.
Summary.—Estimated weight of anterior part of animal; *i.e.*,
    one half of total body weight......................... 3,750 gm.

Quantity of blood circulating (7 per cent of weight)...... 262 cc.
Fluid injected....................................... 260 "
                                              522 "
Blood samples taken for analysis..............about   39 "
Total circulating fluid..........................about 483 ".

Excess dextrose circulating ((1.16–0.05 per cent) × 483 cc.)   5.36 gm.
Dextrose in blood samples taken......................   0.26 "
Total dextrose accounted for......................about   5.62 "
Dextrose injected.................................... 30 "

Amount of dextrose not accounted for, 24.4 gm. or 81 per
    cent of the quantity injected.

The interpretation of these figures is made difficult by several complications. As we injected the same amount of dextrose (4 gm. per kilo) as in the first two series, the dosage of dextrose per kilo was manifestly higher in these experiments since the posterior part of the body was not fed by the circulation. Exactly how much higher cannot be said definitely, but probably the remaining circulation received about twice as much dextrose per kilo as in the experiments on the entire animal. The infusion of Ringer's solution and the occasional use of adrenalin also make these experiments not entirely comparable with the others. However, the first blood sample was always taken after injection of Ringer's solution or adrenalin, so that a comparable initial glycemia was always ascertained.

In these experiments the blood sugar rose greatly; at the end of the injection it reached from 1.35 to 2.41 per cent, an average of 1.93 per cent for eleven experiments. In considering these figures it must be remembered that, as stated above, the dose of sugar per volume of circulating blood was about twice as high as in the other two series. In every experiment there was a rapid fall in the blood sugar after the injection had been finished. Since the samples of blood were not always taken at the same intervals, it becomes necessary to study the experiments in groups. In Experiments 55, 56, and 57 the

TABLE III.

Intravenous Injection of Dextrose into Dogs with Aorta and Vena Cava Ligated near the Diaphragm.

No. of experiment.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.	Blood sugar at end of injection.	Blood sugar ½ hr. after injection.	Blood sugar 1 hr. after injection.	Blood sugar 1½ hrs. after injection.	Blood sugar 2 hrs. after injection.	Muscle before injection.		Muscle after injection.		Sugar not accounted for. Per cent of total amount injected.	
	gm.	cc.							Dextrose.	Polysaccharides.	Dextrose.	Polysaccharides.	By blood.	By blood and muscle.
			per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
LD18......	18.0	90		2.33		1.44							82	82
LD20......	25.6	128	0.15 (3 hrs. later) 0.11	2.03		1.16							87	
LD21*‡...	28.0	140	0.14 (1 hr. later) 0.04	2.34		1.55							80	80
LD28*§...	41.0	205	0.19	2.18		1.58							78	78
LD30*‖...	30.0	150	0.07 (1 hr. later) 0.05	1.65		1.16							82	82
LD31†¶...	41.0	205	0.10	1.65		1.18							83	83
LD35†*...	27.0	135	0.40	1.81		1.34		1.13					86 (1 hr.) 89 (2 hrs.)	
LD45***...	34.0	170	0.33	1.75	1.02						0.49	0.21	74	
LD55......	37.0	176	0.15	1.35	1.70				0.26	0.15	0.36	0.24	91	82
LD56*.....	29.8	144		2.41					0.40	0.18	0.71	0.50	81	49
LD57*.....	36.7	183.7	0.17	1.73	1.38				0.52	0.54	0.80	0.37	91	86
Average.....				1.93†† 1.83‡‡ 2.00§§	1.37	1.34								

272

* Thoracic duct ligated.   † Thoracic duct ligated and thyroid glands removed.

‡ 15 cc. of Ringer's solution injected between first and second blood samples.

§ 100 cc. of Ringer's solution and 1 cc. of adrenalin (in small portions) injected before taking first blood sample.

∥ 110 cc. of Ringer's solution (in small portions) injected between first and second blood samples.

¶ 75 cc. of Ringer's solution injected before taking first blood sample.

** Ether and magnesium sulphate anesthesia. 40 cc. of Ringer's solution injected before taking first blood sample.

** 60 cc. of Ringer's solution and 0.5 cc. of adrenalin injected before dextrose injection.

†† Average of eleven.   ‡‡ Average of last three.   §§ Average of first seven.

TABLE IV.

Intravenous Injection of Dextrose into Whole Dead Animals.

No. of experiment.	Interval between heart stoppage and injection. (min.)	Dextrose injected (4 gm. per kilo). gm.	cc.	Blood sugar before injection. per cent	After injection. Time. min.	Blood sugar. per cent	Time. min.	Blood sugar. per cent	Time. min.	Blood sugar. per cent	Muscle before injection. Dextrose. per cent	Polysaccharides. per cent	Muscle after injection. Time. min.	Dextrose. per cent	Polysaccharides. per cent	Dextrose not accounted for. Per cent of total amount injected. By blood. per cent	By blood and muscle. per cent
LD48	3	21	105		9	1.95	73	3.85†								56	
LD49	21	30	150		30	3.26					0.54	0.15	83	0.88	0.83	30	0
					46	2.83*											
LD51	25	22	110		30	1.54					0.29	0.14	42	0.60	0.21	65	27
LD53	14	18	90	0.02 (?)	37	1.67	67	1.48	157	1.30(1.01)‡	0.33	0.21	161	0.45	0.22	71	62
LD54	10	32	160	0.23	30	2.18	60	2.03	105	1.93	0.38	0.30	126	0.64	0.60	64	6

* This sample of blood was obtained from the aorta after tying the inferior and superior venae cavae.

† This sample of blood was obtained from the portal circulation.

‡ 100 cc. saline were injected in order to get this sample and the figure 1.30 per cent was calculated on the assumption that the saline and blood were intimately mixed.

average percentage of sugar in the blood at the end of the injection was 1.83 per cent, and a half hour later the average was 1.37 per cent—a fall of 0.46 per cent in half an hour. In Experiments 18, 20, 21, 28, 30, 31, and 35, the blood sugar fell from an average of 2.00 per cent at the end of the injection to 1.34 per cent in an hour; in Experiment 57 the fall continued steadily from 1.73 to 1.38 in half an hour, and to 0.82 per cent in 1½ hours. In Experiment 35 a drop in the blood sugar from 1.81 to 1.34 per cent occurred in the first hour after the end of the injection, while during the second hour only a relatively slight decrease took place; namely, to 1.13 per cent.

In every experiment a large percentage of the injected sugar was not accounted for by the sugar present in the circulating blood, from 74 to 91 per cent disappearing from the blood stream during the time of observation. Another way of demonstrating this is by estimating the percentage of sugar which would have been present in the blood if all the injected sugar was still there and comparing this figure with the actual findings, taking into account the volume of blood drawn and of fluid injected. Such estimations indicate that the blood sugar content would be about 7.7 to 7.9 per cent if all the sugar was in the circulating blood. As a matter of fact the highest blood sugar values in all these experiments ranged from 1.35 to 2.41 per cent, or an average of only 1.93 per cent. It is thus evident that the greater part of the injected sugar left the blood rapidly, in fact before the end of the injection. This disappearance of dextrose from the blood stream went on, then, entirely independently of the abdominal viscera. Hence neither the glycogenic activity of the liver nor an excessive consumption of sugar by or in any abdominal organ can account for the greater part of the dextrose which so rapidly leaves the blood after intravenous infusion.

Analysis of Muscle for Carbohydrates.—In a few of these experiments we analyzed skeletal muscles to determine whether sugar had been stored in them. Higher carbohydrates, as well as dextrose were estimated. A few preliminary studies convinced us that the carbohydrate content of normal muscle is not constant. Therefore it was necessary to determine in each experiment the amount of dextrose and higher carbohydrate present in the muscles before and after the injection.

In Experiments 55, 56, and 57 the procedure was as follows: One fore-leg was amputated before the injection of dextrose and immediately placed in the refrigerator.   At the end of the experiment the animal was killed, the other fore-leg removed and also placed in the refrigerator. The muscles were dissected off, put through the grinder twice, and extracted with water in the presence of toluene for about 18 hours in the refrigerator.   The fluids were then pressed out and the residue again extracted with water for about 2 hours.   All fluids obtained were immediately boiled after adding a little acetic acid and filtered through glass wool. The residue, pressing cloth, etc., were thoroughly extracted with boiling water. The combined fluids were then treated with phosphotungstic acid solution while boiling, filtered, neutralized with sodium hydroxide, acidified with acetic acid, and finally concentrated to a definite volume on the water bath.   Estimations of the reducing power were made before and after hydrolysis and the results expressed as dextrose.   Hydrolysis was effected by boiling under a reflux condenser for 1½ hours in the presence of 1.8 per cent hydrochloric acid.

In all three experiments there was an increase in the total carbohydrates (*i.e.*, the amount found after hydrolysis) after the injection. There was, however, some slight difference with regard to the behavior of the two types of carbohydrates. In Experiments 55 and 56 the increase in dextrose is practically the same as that of the higher carbohydrates; namely, 0.10 per cent for dextrose and 0.09 per cent for higher carbohydrates in Experiment 55; and 0.31 per cent for dextrose and 0.32 per cent for higher carbohydrates in Experiment 56. In both experiments the time was half an hour. In Experiment 57, where the time was 1½ hours, there was an increase of 0.28 per cent in the dextrose and a decrease of 0.17 per cent in the higher carbohydrates. The fact that the higher carbohydrates were found to be increased after 30 minutes and decreased after 90 minutes might indicate that the higher carbohydrates were more readily utilized than the lower ones, or that dextrose before being oxidized by the muscle must be converted into a polysaccharide. Our evidence, however, comes from only three experiments, and consequently we cannot attach much importance to it.

Does this increase in the carbohydrate content of muscle account for all the injected dextrose which disappeared from the circulation? Consideration of the following figures will show that we are not entitled to answer this in the affirmative. If we assume that the muscle tissue of the dog constitutes approximately 43 per cent of

the body weight, as in man,[34] and that half the body is reached by the circulating blood, we can estimate roughly the increase in carbohydrates of the muscles. Such calculations show only comparatively small percentages of the injected dextrose. Thus in Experiment LD 55 the amount not accounted for is reduced from 91 to 82 per cent by taking the muscle carbohydrates into consideration; in Experiment LD 57 the reduction is from 91 to 86 per cent; and in Experiment LD 56 it is somewhat greater, namely, from 81 to 49 per cent. From this it seems evident that the anterior muscles in these experiments did not contain all the missing sugar. On the other hand, it must be admitted that one cannot draw definite conclusions from the muscle analyses, for which great accuracy cannot be claimed. Furthermore it is possible that, in these "anterior" animals, loss of dextrose may occur through the tissue spaces into the posterior part of the body, which we have not included in our estimations. Finally we have not examined other tissues, e.g., the brain, lungs, spinal fluid, bone marrow, etc., where the carbohydrates might also have increased in amount.

Thus, while we cannot claim that the experiments on the muscles in anterior animals throw definite light upon the fate of all the dextrose which disappears from the circulation after an intravenous injection, the fact remains that a notable increase in the carbohydrate content of the muscles was found.

Experiments with Intravenous Injections of Dextrose into Whole Dead Animals.

The foregoing experiments brought up the question whether this passage of sugar from the blood into the tissues is a vital process; this led to a series of experiments in which dextrose was injected into dead animals.

Method.—The dogs were prepared as in the experiments in Tables I and III on normal and anterior animals, except that, under light chloroform insufflation anesthesia, three or four ribs were resected on the left side in order to expose the

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[34] Vierordt, H., Anatomische physiologische und physikalische Daten und Tabellen, 3rd edition, Jena, 1906, 44.

heart. Then full chloroform vapor was given until the heart stopped. In some cases one fore-leg was then removed for analysis, all bleeding points being carefully ligated. Continuing the artificial respiration and massaging the heart by hand, we injected the dextrose solution intravenously, much more quickly, however, than in the experiments upon living animals. The rhythmic heart massage was continued for 5 or 10 minutes, and blood samples were taken from the carotid while massaging the heart.

The following is a protocol of one experiment.

Experiment LD 54.—Dog, male; weight 8 kilos.

10.35. Etherized. Intratracheal insufflation begun and chloroform anesthesia produced by this method. Cannulas were inserted in the left jugular vein and right carotid artery. The thorax was opened on the left side, four ribs being resected and the heart exposed. The left fore-leg was then amputated.

12.08. Left fore-leg placed in refrigerator.

12.10. Full chloroform vapor given.

12.20. The fibrillary twitchings of the heart have ceased.

12.23–12.24. Blood taken from the carotid artery, 23.03 gm., 0.23 per cent.

12.30–12.32½. 160 cc. of warm 20 per cent dextrose solution injected into left jugular vein, followed by 3 cc. 0.9 per cent sodium chloride. Heart massaged during the injection.

12.32½–12.36. Massaging of heart continued.

1.02–1.04. Blood taken from carotid, 20.04 gm., 2.18 per cent.

1.31–1.34. Blood taken from carotid, 16.07 gm., 2.03 per cent.

2.17–2.18. Blood taken from carotid, 20.74 gm., 1.93 per cent.

2.39. Right fore-leg removed and put in refrigerator.

Analysis of Muscle of Fore-Legs.

Control muscle: 0.38 per cent before hydrolysis (dextrose).
0.68 per cent after hydrolysis (total carbohydrates),
or 0.30 per cent polysaccharides.

Muscle after dextrose injection: 0.64 per cent before hydrolysis (dextrose).
1.24 per cent after hydrolysis (total carbo-hydrates),
or 0.60 per cent polysaccharides.

Increase in total carbohydrates, 0.56 per cent.

Summary.—Amount of dextrose injected, 32 gm. 1¾ hours after the end of the injection, the blood sugar was 1.93, or 1.70 per cent above its original value. There was estimated to be about 636 cc. of blood in the blood vessels at the time of taking the last blood sample.

Increase in dextrose in blood (636 × 1.70 per cent) ....10.8 gm.

Dextrose removed in blood samples.................. 0.7 "

Total excess dextrose in blood.......................11.5 "

or 36 per cent of amount injected.

Therefore 64 per cent not accounted for by blood.

The amount of muscle was estimated to be 3,300 gm.; that is, 43 per cent of (8,000 gm. (body weight)–340 gm. (amputated leg)).

Increase in total carbohydrates in muscle (3,300 × 0.56 per cent) 18.5 gm.

Total excess dextrose in blood............................. 11.5 "

Total dextrose accounted for by blood and muscle............. 30.0 "

or 94 per cent of the amount injected.

Therefore, 6 per cent not accounted for.

From Table IV, which summarizes the experiments upon whole dead animals, it is seen that the blood sugar content never reached 4.4 to 4.75 per cent, which we estimate would have been the percentage if all the sugar injected had remained in the blood. The highest blood sugar percentage found was 3.85 per cent in the portal blood in Experiment LD 49; the lowest was 1.48 per cent (except the 1.30 per cent of the same experiment, which is not absolutely reliable because of the method used in obtaining this blood sample). The irregularity in the figures of Experiment LD 49 is due to the fact that the various samples were taken from different parts of the circulation. Although one cannot compute the total amount of sugar in the blood in the dead animal experiments with accuracy, it seemed desirable to obtain a basis of comparison with the other series. We therefore give the figures in the column, "Dextrose not accounted for by blood," estimating the blood at 7 per cent of the body weight and using the last blood sugar figure obtained (except in Experiment LD 49 in which the average was used). It is thus seen that from 30 to 71 per cent of the sugar injected was not present in the blood.

In four of these experiments muscle taken before and after the injections was analyzed, and the increase in total carbohydrates indicated that a considerable proportion of injected sugar was to be found in that tissue. In Experiment LD 49 the dextrose content of the muscle rose from 0.54 to 0.88 per cent in 83 minutes, and the higher carbohydrates from 0.15 to 0.83 per cent. This increase in total carbohydrates of the muscle[35] accounted for practically the entire 30 per cent of the injected sugar which was still unaccounted for by the blood. A similar result occurred in Experiment LD 54,

[35] The proportion of muscle in the body was assumed to be 43 per cent of the body weight.

as all but 6 per cent of the injected sugar was accounted for by the blood and muscle analyses together. Here the rise in muscle dextrose was from 0.38 to 0.64 per cent, and in muscle polysaccharides from 0.30 to 0.60 per cent, the time being about 2 hours. In fact this result was the most striking of all, for here the muscle contained about 58 per cent of the injected dextrose. In Experiment LD 51 the amount of sugar still unaccounted for after 42 minutes was reduced to 27 per cent by the muscle carbohydrates, the dextrose increasing from 0.29 to 0.60 per cent, while the higher sugars increased but little; namely, from 0.14 to 0.21 per cent. This was the shortest of the four experiments. In Experiment LD 53 there was very little increase in the dextrose of the muscle, from 0.33 to 0.45 per cent, and none in the polysaccharide content. As this was the longest experiment (2⅔ hours), bacterial activity might be thought of as an explanation of the finding of the smallest increase of carbohydrate in the muscle. The increase in the polysaccharide content of the muscles in three of the four experiments is possibly significant, indicating perhaps a condensation of dextrose, after death, within the muscles.

Experiments with Intravenous Injections of Dextrose into the Anterior Parts of Dead Animals.

In Table V are given the results of four experiments upon dead anterior animals. These experiments were similar to the preceding series except that, before causing the heart to stop beating, the aorta and vena cava were ligated near the diaphragm.

In these experiments we again find high blood sugar values, and again we may compare them with the values which we estimated would exist if all the injected sugar had remained within the blood vessels, making due allowance for fluid injected and for blood samples drawn. The calculations give from 7.3 to 7.9 per cent blood sugar. It is seen that the percentages of sugar are actually much lower; namely, 2.24 to 3.42 per cent, showing that a good deal of the sugar injected into these dead stumps left the circulation rapidly. From Experiments 50 and 58 it would seem that this occurred most quickly in the first 30 to 45 minutes and then proceeded more slowly.

In three of the experiments the muscles were analyzed before and after the injection, and it was found that the muscles were richer in

TABLE V.

Intravenous Injection of Dextrose into the Anterior Parts of Dead Animals.

No. of experiment.	Interval between heart stoppage and injection.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.	After injection.		After injection.		Muscle before injection.		Muscle after injection.			Dextrose not accounted for. Per cent of total amount injected.	
					Time.	Blood sugar.	Time.	Blood sugar.	Dextrose.	Polysaccharides.	Time.	Dextrose.	Polysaccharides.	By blood.	By blood and muscle.
	min.	gm.	cc.	per cent	min.	per cent	min.	per cent	per cent	per cent	min.	per cent	per cent	per cent	per cent
LD47*	2	27.0	135		15	3.27	43	3.07						55	
LD50*	42	23.0	115		18	3.42	68	2.92†	0.47	0.57	128	1.03	0.57	58	30
LD58	0	31.7	156	0.09	21	3.39	70	2.24	0.44	0.39	88	0.74	0.42	73	56
LD59	2	36.0	180	0.16	30	2.32	60	2.34	0.52	0.43	81	0.84	0.51	71	52

* Thoracic duct ligated also.

† This sample of blood was obtained from the aorta after tying the inferior and superior venæ cavæ.

dextrose after the injection than before. The percentage of muscle dextrose rose from 0.47 to 1.03, from 0.44 to 0.74, and from 0.52 to 0.84 per cent, respectively. The figures given in the last two columns of the table were obtained on the assumption that the injected sugar reached only half the body, and further that this half contained the same proportions of blood and muscle as the whole body; namely, 7 and 43 per cent, respectively. The first of these two columns shows that a large proportion of the injected sugar had disappeared from the circulation. In Experiment LD 47 about 55 per cent was estimated to have left the blood in 15 minutes. In the other three experiments, each lasting about 70 minutes, the amount of sugar lost from the blood was about 58, 71, and 73 per cent. From the last column of the table we see that even when we deduct the sugar found in the muscle tissue there is still from 30 to 56 per cent not accounted for. In none of these experiments were we able to account for practically all the injected sugar by the muscle and blood analyses, as in two of the experiments on the whole dead animals.

The increase in the higher carbohydrates of the muscle in these experiments is negligible. In Experiment LD 50 there was no increase; in Experiment LD 58 the increase was from 0.39 to 0.42 per cent; and in Experiment LD 59 from 0.43 to 0.51 per cent. These results are in contrast with those found on the entire dead animals (Table IV) in which there was a considerable increase in the muscle polysaccharides in two experiments and a small increase in another. This difference may perhaps be brought into connection with the observation made by Levene and Meyer;[36] namely, that the pulp or juices of various tissues of the dog are able to convert dextrose into a higher carbohydrate if they are activated by spleen juice. In the anterior animal such a mixture of the products of the spleen and tissues by means of an artificial circulation is excluded.

DISCUSSION.

Large amounts of dextrose were injected intravenously into normal dogs. A variable proportion (an average of about 60 per cent) was eliminated by the kidney during the injection and the 90 minutes

[36] Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1912, xi, 353.

which followed.   By the end of this time the percentage of sugar in the blood had fallen to, or nearly to, the level found before the injection. Consequently about 40 per cent of the injected sugar was not accounted for by the blood and urine analyses.   When the same amount of sugar was injected into nephrectomized dogs, the blood sugar, while rising somewhat higher in the course of the experiment, fell to its original level as quickly as in the normal animals.   Therefore nearly all the sugar had left the blood in the same length of time without the aid of the kidneys.   When dextrose was injected intravenously into animals which had practically no circulation posterior to the diaphragm, the blood sugar did not reach the height which it would have attained if all the sugar had remained in the blood vessels. Since the dosage, however, was based on the weight of the entire animal, and as these dogs had only about half their circulation, the dosage was really about twice as great as in the first two series.   For this reason an exact comparison cannot be made.   Nevertheless, it was seen that the blood sugar gradually fell and that from 74 to 91 per cent of the injected sugar had left the circulation during the injection and the after-period, which was usually not more than 1 hour.   Even in dead animals the blood sugar did not rise to nearly the percentage it would have reached if the injected sugar had remained in the blood vessels.   Here, too, the percentage gradually fell, showing that from 30 to 71 per cent had left the blood without any vital forces taking part.   In the last series, the dead anterior animals, a similar result occurred; from 55 to 73 per cent of the injected sugar could not be accounted for by the blood.

All these experiments show that the injected sugar disappeared quickly from the circulation.   The first question that arises is: Is all this sugar rapidly burned up by the organism?   The oxidation of carbohydrate in the animal body is usually tested by determining the respiratory quotient, a rapid rise of which to or near to unity being taken as an indication of sugar combustion.   This has not been done by us, but it is well known that after the ingestion or injection of dextrose into the normal animal a rise in the respiratory quotient occurs.   There is, however, no reason to assume that a nephrectomized animal will burn dextrose twice as fast as a normal animal. Yet this would have to be the assumption if we were to explain the

results of our normal and nephrectomized series on the basis of a rapid combustion. Tangl,[37] indeed, has found that after nephrectomy the gas exchange is diminished for 3½ to 4 hours, after which it begins to rise slowly. It therefore seems that a nephrectomized animal does not have a greater metabolism than a normal one during our experiments and probably does not oxidize more sugar. Another argument against rapid combustion is the disappearance of large amounts of sugar from the blood of dead animals. Here it can hardly be a question of increased metabolism.

The transformation of dextrose into glycogen in the liver is undoubtedly only a small factor in the disposal of intravenously injected glucose. This is evident from the work of Harley[18] and, more especially, of Freund and Popper.[24] The latter analyzed lobes of the liver before and after dextrose injection, and, when using a dosage like ours, found no increase in the glycogen content. Our experiments upon anterior animals, in which dextrose disappeared from the blood stream when all the abdominal viscera were cut off from the circulation, demonstrate clearly that the glycogenic function of the liver is not of great significance in tracing the fate of intravenously injected dextrose.

Undoubtedly one of the most important factors is the passage of the sugar into the surrounding soft tissues. In two experiments upon the dead entire animal practically all the dextrose not found in the blood was accounted for by the increased amount of sugar in the muscles. In the other experiments upon dead whole animals and dead anterior animals, from 9 to 38 per cent was estimated to be present in the muscles, and in three living anterior animals the estimations for muscle sugar were 5, 9, and 32 per cent of the amount injected. Evidently, a large and variable proportion of the injected dextrose simply passes through the capillaries into the muscle tissue. No doubt, the same process occurs in other parts of the body, more especially in all the soft tissues. The rapid disappearance of sugar from the blood is therefore, to a large extent at least, not difficult to explain.

However, the passage of dextrose into the tissues is not the only cause for its disappearance, for even in the dead animal experiments

[37] Tangl, F., *Biochem. Ztschr.*, 1911, xxxiv, 1.

we could not account for all the injected sugar in any of the anterior experiments or in two of the four experiments upon the dead whole animal. It is possible that some part of the dextrose is converted into higher carbohydrates in certain tissues. In fact some evidence, presented in the foregoing pages, indicates that this does occur in the muscles. In the living anterior experiments an increase in the higher carbohydrates of muscle occurred in two out of the three cases in which the muscle was analyzed. The same result was obtained in the dead whole animals in three out of four tests, while in the dead anterior animals there was little or nb formation of polysaccharides. Apparently a condensation does occur in the muscles. But this does not account for any more of the sugar which has disappeared from the blood because it has already been included in our estimations. It may be that such a reaction occurs to the same or to a greater extent in other tissues and, in that way, a large part of the missing fraction would be accounted for.

The fact that in the dead whole animal a considerable condensation to a higher carbohydrate occurred in the muscles, while in the anterior ones very little was evident, suggests that this reaction may be influenced by an intra-abdominal organ. In the living anterior animals, however, a similar polymerization was found. To harmonize this with the above suggestion one would be obliged to assume that the almost insignificant amount of circulation still existing posterior to the diaphragm in living anterior animals suffices to bring from the abdomen the substance required to aid in the condensation. In the dead anterior animals the artificial circulation maintained at times by massaging the heart would not be enough to bring this about.

As other investigators have suggested, it is possible that a part of the injected dextrose is broken down to lower incompletely oxidized compounds. It would therefore not be entirely burned and at the same time, it would not be found as dextrose. We have no evidence to offer on this point.

SUMMARY. .

1. As has been found by other investigators, when a large amount of dextrose is injected intravenously into a normal dog it disappears from the circulating blood in about 90 minutes after the end of the

injection.   Varying amounts (an average of 60 per cent) are excreted in the urine.

2. Even in nephrectomized animals the same quantity will leave the circulation in the same length of time as in normal animals.

3. This phenomenon seems to be, at least to a great extent, independent of vital processes, since dextrose, after intravenous injection into dead animals, is found to leave the blood rapidly.

4. The phenomenon is independent of the important abdominal organs, for it also occurs in animals (living or dead) in which the aorta and inferior vena cava have been ligated near the diaphragm, thus abolishing most of the circulation posterior to the diaphragm.

5. The fact that a considerable amount of the sugar passes from the circulation into the surrounding tissues was established by finding an increase in the carbohydrates of the muscle tissue.   This was done in the case of the living anterior animals and in the whole and anterior dead animals.   In most of these experiments there was also evidence of the formation of polysaccharides in the muscle tissue.

THE INTRAVENOUS INJECTION OF MAGNESIUM SUL-PHATE FOR ANESTHESIA IN ANIMALS.

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The effect on animals of intravenous injections of magnesium sulphate was investigated by us from a general experimental point of view about ten years ago.[1] The use of this salt for practical purposes of anesthesia in human beings was first studied by Haubold and Meltzer by the intraspinal method.[2] About two years ago a combination of subminimal doses of magnesium sulphate intramuscularly and ether by intratracheal insufflation was found by the present writers[3] to be effective in animals, and by Peck and Meltzer and also Elsberg and Meltzer in human beings. The use of magnesium sulphate by intravenous injection was in general discouraged by one of us.[4] However, a series of experiments made by the present writers with intravenous injection of magnesium sulphate in cases of experimental tetanus,[5] and the meager but satisfactory experience which Kohn[6] and Straub[7] had with the employment of this method in cases of tetanus in human beings, induced us to take up the experimental study in animals of the employment of magnesium sulphate by intravenous injection for the purpose of producing anesthesia. This was done as a preliminary test for the admissibility of

[1] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905–06, xv, 387.

[2] Haubold, H. A., and Meltzer, S. J., *Jour. Am. Med. Assn.*, 1906, xlvi, 647.

[3] Meltzer, S. J., and Auer, J., *Proc. Soc. Exper. Biol. and Med.*, 1912–13, x, 159; *Zentralbl. f. Physiol.*, 1913–14, xxvii, 632.

[4] Meltzer, S. J., *Berl. klin. Wchnschr.*, 1915, lii, 261.

[5] To be published later.

[6] Kohn, H., *Berl. klin. Wchnschr.*, 1915, lii, 89.

[7] Straub, W., *München. med. Wchnschr.*, 1915, lxii, 341.

studying the exclusive use of intravenous injections of magnesium sulphate as a means of producing, or at least inducing, anesthesia in human beings. We wish to record a few abbreviated protocols of these experiments.

<div align="center">EXPERIMENTAL.</div>

The experiments were made on dogs. The left external jugular vein was exposed under local anesthesia by ethyl chloride, a cannula introduced, and magnesium sulphate in an $\frac{M}{4}$ solution injected through the cannula from a burette. The reaction of the animals was tested in various ways, as indicated in the protocols. When the respiration appeared to be shallow, pharyngeal insufflation[8] was employed either temporarily or throughout the entire experiment. In some instances intratracheal insufflation was given, especially for the purpose of testing the possibility of introducing the intratracheal tube without using any other anesthetic and while the animal was still breathing spontaneously. At the end of the experiment either a small quantity of a calcium chloride or sodium sulphate solution was injected, or no further injection was given.

Experiment 1.—Black and white female fox-terrier; weight 4,600 gm.

10.20. On electric warming pad at medium. Clip hair of neck; used ethyl chloride as local anesthetic for exposing and inserting cannula in external jugular vein.

10.45. Rectal temperature 38.8°C.

10.50. Start infusion of $\frac{M}{4}$ magnesium sulphate into jugular vein.

10.55. Operation completed.

11.00. 5 cc. Lid reflex prompt and strong.

11.04. 14 cc. Slightly restless.

11.05½. Lid reflex prompt; active expiration, of good strength.

11.08. 20.5 cc. Lid reflex prompt and strong. Respiration good, fairly rapid, active expiration.

11.11. 26 cc. Respiration fairly rapid; quiet; lid reflex prompt and strong.

11.13. 33 cc. Respiration slower, with active expiration; no sound. Heart slow.

11.15. Catheter F. 19 inserted into trachea·with ease; start air insufflation.

11.17. 40.5 cc. Lid reflex slight.

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[8] Meltzer, S. J., *Jour. Am. Med. Assn.*, 1913, lx, 1407; *Berl. klin. Wchnschr.*, 1915, lii, 425.

11.18. Very slight inspiration, slow rate; able to move head slightly.

11.20. 44.5 cc. Very shallow respiration on stopping insufflation.

11.22. 46.5 cc. Lid reflex fair.

11.23. Occasionally spontaneous, fair expirations during insufflation.

11.24. 50 cc. injected. Stop. Very slight spontaneous shallow respiration; slow rate. Lid reflex slight.

11.25. Injected through venous cannula 15 cc. $\frac{M}{8}$ calcium chloride into jugular vein. Respiration began promptly. Injection lasted about 1 minute. Suture wound after wiping with diluted tincture of iodine. Remove catheter. Animal trots away at once, slightly unsteady at first; wags tail, obeys command.

12.20. Runs about with ease.

2 days later, 10 a.m. Dog lively and active, jumps about, barks, behaves like a normal dog.

Next day. Good condition.

The intravenous injection of an $\frac{M}{4}$ solution of magnesium sulphate was given at a slow rate. After injecting 33 cc. in 23 minutes the intubation for intratracheal insufflation was performed with ease, while usually a great deal of ether has to be given to accomplish this purpose. A total of 50 cc. of magnesium was given in 34 minutes. The spontaneous respiration was then very shallow. After injecting 15 cc. of $\frac{M}{8}$ calcium chloride the respiration recovered promptly and the animal would execute satisfactory voluntary movements.

Experiment 2.—Black and white mongrel, male; weight 5,850 gm.

Preliminary preparations as in Experiment 1.

11.37. Start infusion of $\frac{M}{4}$ magnesium sulphate into jugular vein.

11.41. 9 cc.

11.44. 18 cc. Moves head, lid reflex strong. Barks sleepily.

11.46. 22 cc. Respiration more rapid, shallower; no reaction to pricking skin with needle; lid reflex strong.

11.50. 36 cc. Fair, slow respiration with active expiration. Lid reflex prompt. Occasional slight general motions. Heart slowed.

11.52. 39.5 cc. Respiration slow, moves head.

11.55. 46 cc. Very slow respiration, lid reflex fair; heart slow.

11.57. 50 cc. Respiration very slight; start pharyngeal insufflation. Pulse improved.

12.02. 54.5 cc. Stop insufflation to change stomach tube; no respiration seen.

12.04. 55.5 cc. Heart 24 to $\frac{1}{4}$ minute, small, regular, soft.

12.06. 56.5 cc. Breathes spontaneously; no reaction to pricking skin with needle.

12.09. 61 cc. Tracheal catheter inserted; slow spontaneous respiration, fair depth, some active expiration; lid reflex slight, moves head.

12.12. 64 cc. Legs relaxed; spontaneous respiration fair; lid reflex slight.

12.15. Pulse 27 to ½, regular, strong (femoral); fair spontaneous respiration. Stop insufflation. 65.5 cc. injected. Respiration gradually improves and becomes good, deep. Respiration moderately slow; start air again.

12.16. Excellent respiration; no lid or corneal reflex. Tongue pink.

12.20. Respiration easy, more rapid; no lid or corneal reflex. 70 cc. injected.

12.22. Moves head; increase magnesium flow slightly.

12.23. 73 cc. Respiration slower, but good depth. Femoral pulse 22 to ½, respiration good depth.

12.27. No reaction to pricking skin. 78.5 cc. Stop magnesium.

12.28. 8 cc. of $\frac{M}{8}$ calcium chloride into jugular vein. Respiration greatly improved and more rapid. Wound sutured. Placed on floor, holds head up; front legs spread, do not support body; licks jaws; moves tail on pressure. Pays attention to call and wags tail.

12.35. Able to walk about; tail erect.

2.10. Walks about normally.

Next day. Good condition.

The first 50 cc. injected in 20 minutes, about 2.5 cc. per minute, nearly completely abolished respiration. Pharyngeal insufflation was then started and exerted immediately a good effect. From 12.02 to 12.06 only 2 cc. were injected, equal to 0.5 cc. per minute; spontaneous respiration returned, but no reaction to pricking, and intubation of catheter was easily executed. Thereafter the rate of inflow was kept fairly low. Spontaneous respiration was continually present but was generally slow. There was no lid reflex and no reaction to pricking. After injecting about 78 cc. of magnesium in 50 minutes a quantity of 8 cc. of $\frac{M}{8}$ calcium chloride was injected; respiration improved at once. Voluntary movements, however, returned gradually.

Experiment 3.—Male; weight 7,500 gm.

Was fed previous to experiment. Preparation as in previous experiments.

2.27. Rectal temperature 38.7°. Femoral pulse 33 to ½, regular.

2.33. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

2.37. 7 cc. Respiration deeper; swallows occasionally.

2.40. Pulse softer, 38 to ½, regular; respiration more rapid with strong active expiration. 14 cc.

2.41. Vomited yellowish brown fluid.

2.43. Vomited large amount of yellow fluid with masses of meat.

2.44. 22 cc. Lid reflex prompt; occasional moderate struggle.

2.48. 32 cc. Moderate barks. Lid reflex strong.

2.49. 38.5 cc. Respiration chiefly expiratory. Tracheal catheter inserted; animal shows resistance. Catheter F. 21; constant air stream with remission. Lid reflex strong.

2.54. 44 cc. No reaction to pricking skin with needle.

2.55. 45 cc. Stop insufflation; practically only one inspiration and a number of weak abdominal contractions appeared during the intermission. Lid reflex strong. Able to move head moderately. Occasionally a sharp expiratory movement of abdomen. Started intratracheal insufflation again.

3.06. Remove tracheal catheter; start pharyngeal insufflation, tube in stomach.

3.10. 57 cc. Moves head vigorously; femoral pulse regular, soft.

3.13. 59.5 cc. Legs limp. Lid reflex good.

3.22. 73 cc. Open abdomen, rub peritoneum above liver; no motions.

3.23. 76 cc. Lid reflex fair.

3.26. 80 cc. Stop insufflation; no definite respiration, some expiratory contractions. Start insufflation. Abdomen closed.

3.31. 80 cc. No definite respiration on stopping insufflation. Good lid reflex. Pupils wide.

3.35. 90 cc. Stop magnesium. Lid reflex fair. Stop air; 3 slight respirations; start insufflation.

3.38. Slight, slow, shallow respiration on stopping pharyngeal insufflation. Femoral pulse 18 to $\frac{1}{2}$, regular, fair tension.

3.40. 10 cc. of $\frac{M}{8}$ calcium chloride into jugular vein. Deep, slow respirations begin.

3.43. Pulse 29 to $\frac{1}{2}$, regular, strong tension; no reaction to pricking skin with needle. Lid reflex strong; pupils wide. Neck wound closed. Placed on floor, attempts to get up.

3.44. Gets up after a few trials.

3.55. Lies on side; no response to pressure on toes; wags tail; tolerates probe in nose for a short time. When placed on feet walks away fairly steadily, then lies down again.

4.25. As before; no response to pressure on legs; lies on side usually. Raises head on call; walks when placed on feet. Lid reflex prompt; drinks some water. Killed later by chloroform.

This animal was fed about 3 hours before the experiment was started. After 14 cc. of the magnesium solution were injected (in 7 minutes) the animal vomited. This indicates the central action of magnesium sulphate. After 45 cc. were injected (in 22 minutes) there was practically no spontaneous respiration, although the lid reflex was strong and the animal was able to move its head. The failure of the respiration in this case was undoubtedly due to the in-

hibitory action of the magnesium sulphate upon the respiratory center, and not to a paralysis of the motor nerve endings of the respiratory muscles, which, as a rule, remain excitable longer than the other skeletal muscles. After 80 cc. of the magnesium solution (in 58 minutes) the abdomen was opened and the sensitive parts of the parietal peritoneum were rubbed without eliciting any reaction, although the lid reflex was still good. This animal received 90 cc. of the magnesium solution in 62 minutes. 3 minutes after stopping the injection the animal had only slow and shallow respirations. The injection of 10 cc. of $\frac{M}{8}$ calcium chloride deepened the respirations; it exerted also, fairly promptly, a favorable effect upon the general motility of the animal. But the return of reactions to a probe inserted into the nose and to other sensory stimuli was slow.

Experiment 4.—Wolf hound, female; weight 6,700 gm.

Preliminary preparations same as in Experiment 1.

2.42. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

2.43. 3 cc.

2.46. 10.5 cc. Lid reflex prompt, sustained. Respiration faster; pulse fuller, faster, 33 to $\frac{1}{4}$. Respiratory irregularity of pulse rhythm practically gone.

2.48. 16.5 cc. Respiration 18 to $\frac{1}{4}$, fair depth, slight active expiration.

2.50. 22 cc. Lid reflex prompt, but no longer sustained closure.

2.52. 28 cc. Respiration good, 14 to $\frac{1}{4}$; femoral pulse 30 to $\frac{1}{4}$, good volume and tension; no irregularity.

2.55. 36 cc. Respiration slower; pain sensation of skin abolished.

2.56. 39 cc. Respiration slower, but good depth; pulse irregular. Start pharyngeal insufflation. Stomach tube.

2.58. 46 cc. Lid reflex prompt but slight. Pupils wide.

3.00. 47 cc. Open abdomen and rub diaphragm; no motion of any kind.

3.02. Stop insufflation; very slight shallow respiration; start air; lid reflex a slight flick. Pulse 36 to $\frac{1}{4}$, regular, small volume and tension. Rub peritoneum; no sign of movement; legs limp.

3.07. 51 cc. Lid reflex slightly better; rubbing peritoneum causes no motion of any kind. No knee jerk.

3.08$\frac{1}{2}$ 52.5 cc. Stop insufflation; 16 very slight respirations per $\frac{1}{4}$. Rub diaphragm and parietal peritoneum: no motion. Legs limp. Lid reflex fairly sustained now (closure).

3.15. 56.5 cc. Femoral pulse 32 to $\frac{1}{4}$, regular, small, and soft.

3.17. 57 cc. Stop air; no respiration in 35 seconds. Start air. Lid reflex slight; pupils moderately contracted.

3.21. 58.5 cc. Rub peritoneum and diaphragm; slight motion of leg, but no other perceptible movement. Spontaneous respiration noticeable during insuffla-

tion. Stop air; 24 shallow respirations per ↓. Start air. Pulse 32 to ↓, fair volume and better tension now. Lid reflex prompt but weak.

3.25. 59.5 cc.

3.26. Moves leg slightly.

3.29. 61 cc. On rubbing peritoneum and diaphragm no motion, but later made vigorous movements with head and leg. (No apparent relation to stimulus; rubbing repeated; no motion.)

3.31. Some strong movements of head and legs. Lid reflex flick, not sustained. Stop air. Respiration spontaneous, 15 to ↓, good depth. Stop insufflation entirely.

3.33. 63 cc. of magnesium. Stop. Excellent respiration. Suture abdominal wound. Ligate jugular vein and suture wound in neck. No calcium chloride given.

3.36. Placed on floor; attempts to walk; hind legs spread; raises head and looks about.

3.43. Lying on side; placed on legs; walks about; lies down again shortly. Withdraws legs fairly promptly when pressed; walks away when tail is pressed; no sign of pain.

4.00. Walks about. Rectal temperature 36.4°; pulls away leg when toes are pressed.

4.38. Killed by chloroform.

During the first period of the experiment (about 18 minutes) the inflow of the magnesium solution occurred at a rate of about 2.9 cc. per minute. After the injection of 36 cc. (in 13 minutes) skin sensibility was abolished; the respiration, though slower, was good and there was even an occasional struggle. The pharyngeal insufflation was started before there was any necessity for it. After the injection of 47 cc. (in 18 minutes) the abdomen was opened and the peritoneum rubbed without any reaction. About this time, however, the spontaneous respiration was shallow, lid reflex slight, and the legs were limp. In the following half hour the rate of injection was considerably reduced—about 16 cc. in 33 minutes. The loss of sensibility lasted for about half an hour longer. The spontaneous respiration returned perceptibly sooner. Altogether 63 cc. of magnesium sulphate were injected in about 51 minutes. Then, when the magnesium sulphate injection was stopped and the insufflation discontinued, the spontaneous respiration immediately appeared to be excellent. No calcium chloride was given. After placing the animal on the floor, motility and sensibility returned fairly soon. The quan-

tity of magnesium given in this experiment was not large and the rate of injection slowed down considerably during the latter part of the experiment. The recovery here was prompt and without the aid of calcium chloride.

Experiment 5.—Bull terrier, female; weight 7,600 gm.

Preparation the same as in previous experiments.

Rectal temperature 40.2°. Femoral pulse 30 to $\frac{1}{4}$, small, regular, good tension. Respiration slow, 12 to $\frac{1}{4}$, with active expiration.

10.58. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

11.04. 17 cc. Lid reflex prompt but not sustained; pupils wide. Respiration less deep, slow. Swallows occasionally.

11.06. 25 cc. 14 respirations to $\frac{1}{4}$, good depth, moderate active expiration.

11.07. 29 cc. Lid reflex prompt and sustained. Quiet.

11.08. 35 cc. Pulse small, fairly soft, 38 to $\frac{1}{4}$, regular. Respiration good depth.

11.10. 43 cc. Respiration slow, less deep but still good, 7 to $\frac{1}{4}$. Slow magnesium inflow.

11.13. 50 cc. Respiration improved.

11.15. 53 cc. Magnesium inflow slowed. Lid reflex fairly prompt.

11.18. 56 cc. Open abdomen; rub peritoneum and diaphragm; no motion of any kind. Respiration good, more rapid than before.

11.20. 58.5 cc. Lid reflex slight; respiration rapid, 36 to $\frac{1}{4}$, next count 28 to $\frac{1}{4}$; legs relaxed. Expose left sciatic nerve; no motion at first, later moderate general movements.

11.25. 71 cc. Respiration excellent.

11.29. 86 cc. Respiration much shallower and slower. Start pharyngeal insufflation.

11.32. 98 cc. No lid reflex.

11.34. 102 cc. No definite respiration.

11.37. 104.5 cc. Stimulated left intact sciatic with Petzold inductorium. At coil distances of 200 and 120 mm. no reaction elicited. At 80 mm. respirations appeared during stimulation; left toes moved slightly; also weak general motions and movements of tail. Rub peritoneum and diaphragm; no response.

11.42. 107 cc. Stop pharyngeal insufflation; slight spontaneous respiration present; start pharyngeal insufflation; increase magnesium flow slightly. Limp. No lid reflex; pupils very wide.

11.47. Abdomen and thigh wound sutured. 116 cc. No lid reflex.

11.49. 117 cc. Stop magnesium. Spontaneous respiration very slight; pharyngeal insufflation necessary.

11.50. 60 cc. $\frac{M}{4}$ sodium sulphate into jugular vein. Respiration improved promptly. Stop pharyngeal insufflation.

11.53. Pulse good. Suture neck wound, lid reflex slight.

11.56. Placed on floor, holds head up for short time, then rests it on floor; cannot stand. Wags tail when called.

12.01. Pressure on tail and toes; moves head towards tail, draws away foot.

12.05. Lid reflex very slight, pupils wide. Able to get up but prefers to squat or lie down.

1.55. Walks about readily, keeping left hind leg lifted (left sciatic nerve had been exposed), no staggering; lid reflex prompt and sustained. Pupils well contracted. Urinated large amount; first time since injection.

4.45. Walks about easily when placed on feet (staid in one place since last note); no more urine passed. Killed by chloroform.

This dog had from the start a slow respiration although its temperature was higher than normal. During the first 10 minutes of the magnesium infusion 3.5 cc. per minute were injected, more than in any of the animals in previous experiments. There were no struggles. After injecting 56 cc. the abdomen was opened and the parietal peritoneum rubbed without any reaction, while the respiration was good and even more rapid than before. After 102 cc. no definite respirations were present, and after 104 cc. the motor nerve endings were affected. The inflow was then reduced—only 13 cc. in 12 minutes. Altogether 117 cc. were injected in 51 minutes. There was practically no spontaneous respiration when the infusion of the magnesium solution was discontinued. However, the respiration improved within 1 minute after the injection of 60 cc. of $\frac{M}{4}$ sodium sulphate. The general motor and sensory depression seemed also favorably affected by this injection.

Experiment 6.—Black male; weight 7,500 gm.
Preliminary preparation as in Experiment 1.

3.13. Start $\frac{M}{4}$ magnesium sulphate into jugular vein.

3.17. 12 cc. Respiration 8 to $\frac{1}{4}$. Good depth. Femoral pulse 29 to $\frac{1}{4}$, regular, good volume and tension.

3.18. 15 cc. Lid reflex prompt and strong, pupil moderately dilated. Dog quiet.

3.20. 25 cc. Good respiration, 12 to $\frac{1}{4}$, active expiration stronger. Femoral pulse 32 to $\frac{1}{4}$, regular, good tension. Barks.

3.22. 31 cc. Lid reflex prompt and sustained; pupils wider.

3.23. 34.5 cc. Pain abolished; opening of peritoneum; respiration easy, good depth and frequency. Abdomen relaxed.

3.25. 46 cc. Rub peritoneum and diaphragm; no movement. Lid reflex prompt and sustained; respiration slow, good depth, 11 to $\frac{1}{4}$.

3.27. 50 cc. Blood bright red. Slow magnesium inflow.

3.28. 51 cc.   Rub peritoneum of diaphragm; no movement.   Lid reflex weak.

3.30. Pulse 30 to ½, regular, good volume and tension.

3.31. 53.5 cc.   Slight knee jerk.

3.32. 55 cc.   Moved legs; respiration faster and deeper.   Rub peritoneum and diaphragm; no immediate effect, after a few seconds rapid respiration with moderate strength.   Increase magnesium inflow.

3.34. 61 cc.   Lid reflex a mere flick; rapid respiration with active expirations in short group, then easy respirations without active expirations.

3.39. Respiration slow, good depth with active expiration.

3.42. 85 cc.   Blood a little darker; respiration shallower.

3.44. Start pharyngeal insufflation.

3.46. Pulse 20 to ½, fair volume and tension, regular.   No lid reflex.   Rub peritoneum and diaphragm; no movement.   Legs limp; no knee jerk.

3.52. No spontaneous respiration on stopping insufflation.

3.55. Pupils well dilated but not maximal; no lid reflex.   103 cc.   Pulse small, 25 to ½.

4.00. 104.5 cc.   No lid reflex.   Rub peritoneum; no movement; no knee jerk.

4.05. No spontaneous respiration on stopping insufflation; pulse weak.   Start insufflation again.

4.07. No movement on rubbing peritoneum and diaphragm.   106 cc.   Stop magnesium.   No lid reflex.   Injected 60 cc. $\frac{M}{4}$ sodium sulphate into jugular vein.

4.10. Femoral pulse 25 to ½, small, regular, better tension.   Rubbing peritoneum and diaphragm; no movement.

4.11. Stop insufflation; slow respiration, getting deeper; start insufflation again. No lid reflex; pupil wide.

4.13. Rub peritoneum; no movement.   Legs limp; no knee jerk.

4.17. Suture abdomen and neck wound and stop insufflation.   Pulse 27 to ½, regular, good volume and tension; 4 respirations to ½, good and deep, no active expiration; no lid reflex, pupils wide; 36.4°.   Placed on floor; cannot stand, lies on side.   Pain sensation fair; looks about and wags tail.

4.25. No lid or corneal reflex; wags tail when called; feeble knee jerk; on moderate pressure of toe pads, no movement.   When lifted and placed on floor front legs bear body weight, but not the hind legs.   Pupils widely dilated.

4.30. Respiration easy, good depth, 14 to ½, no active expiration.   Femoral pulse 29 to ½, regular, good volume and tension.   Lies on side, wags tail.   Very slight lid reflex.

4.45. Sits up on haunches, but does not walk about; lid reflex fairly good. Passed small amount of urine.

5.00. Walks about, no weakness; lid reflex prompt and sustained; pupils still wide.   Killed with chloroform.

This strong dog received in the first 10 minutes about 35 cc. of the magnesium solution.   There was very little excitation, and at the

end of this period the skin of the abdomen could be incised to the peritoneum without any reaction. In the following 2 minutes 11 cc. were infused, and the sensitive parts of the parietal peritoneum were energetically rubbed without producing any reaction, while respiration was still good and the lid reflex prompt and sustained. The rate of injection was now reduced, and the spontaneous respiration kept up efficiently for some time. After 19 minutes during which time about 44 cc. were injected (a little less than 2.5 cc. per minute) pharyngeal insufflation was started. In the next 23 minutes only about 16 cc. were injected (about 0.7 cc. per minute). During this period there were no spontaneous respiration, no lid reflex, no knee jerk, and the legs were limp; finally the pulse became weaker. Altogether 106 cc. of the magnesium solution were injected in 54 minutes. At the end of the magnesium injection no calcium chloride was given, but, as in the previous experiment, 60 cc. of sodium sulphate in $\frac{M}{4}$ solution were injected intravenously. The effect of the injection in this experiment, however, was in no way striking. The respiration did not improve at once and the insufflation had to be continued for about 10 minutes longer. The fact should be borne in mind that in this experiment the rate of injection of the magnesium sulphate during the first half of the infusion period was considerably greater than in any of the other experiments.

In addition to the foregoing experiments we wish to record briefly the exceptional course of one of the experiments. This dog had an irregular heart beat and its extremities were rigid before the experiment was begun. There was no spontaneous respiration after injecting 44 cc. of magnesium solution (in 14 minutes), while the peritoneum remained sensitive and the lid reflex active during most of the injection period. The animal received 72 cc. in 57 minutes. The pulse was small and often weak during the last half hour. At the end of the magnesium injection 10 cc. of $\frac{M}{8}$ calcium chloride were injected without restoring the spontaneous respirations. A few minutes later 5 cc. more of the calcium chloride brought on some weak respirations, but the heart stopped soon after and the animal died.

Here was a case in which calcium did not restore the respiration which had been abolished by magnesium; on the contrary, it was perhaps instrumental in accelerating cardiac death.

SUMMARY AND CONCLUSIONS.

These experiments justify the following general conclusions.

By the intravenous injection of $\frac{M}{4}$ magnesium sulphate into dogs at a certain rate, a stage can be reached where the abdominal walls are completely relaxed and when section of the abdomen and stimulation of sensitive parts of the parietal peritoneum do not produce pain or elicit any reaction of the animal. At the same time spontaneous respiration may still be maintained within normal limits and the lid reflex be fair or even normal. In this stage intratracheal intubation for artificial respiration can be easily accomplished. This stage may be attained in 12 to 14 minutes when the rate of injection is about 3 cc. per minute. When this stage is once attained the rate of injection should gradually be reduced, otherwise, sooner or later, spontaneous respiration will be abolished, and by a further maintenance of the rate of injection all the skeletal muscles may become paralyzed.

When the injection of magnesium is continued for a longer period, the paralytic effects of the magnesium injection will set in, even when administered at a slow rate.

The paralysis of the respiratory function is readily met by intrapharyngeal insufflation, which is easily executed even without training in this procedure, or by the method of intratracheal insufflation, if executed by one trained in its management.

When the respiration of the animal is accomplished by insufflation, the paralytic effect of the magnesium may be abolished fairly rapidly by an intravenous injection of about 10 cc. of an $\frac{M}{8}$ calcium chloride solution; or it may disappear slowly, after the infusion of the magnesium solution is discontinued for some time. The latter mode of disappearance may be favorably accelerated by an intravenous infusion of 60 to 100 cc. of an $\frac{M}{4}$ solution of sodium sulphate.

The production of anesthesia by intravenous injection of magnesium sulphate should not be undertaken unless an apparatus for intrapharyngeal insufflation is at hand, because in exceptional cases the disappearance of spontaneous respiration may be one of the earliest consequences of the magnesium injection.

The injection of calcium chloride should not be employed in cases in which the subject shows cardiac insufficiency. In such instances, moreover, injections of magnesium should not be used for the purpose of anesthesia; at least not until greater experience has been acquired in the employment of this method.

AN EXPERIMENTAL STUDY OF THE ADDITIVE AND ANTAGONISTIC ACTIONS OF SODIUM OXA- LATE, AND SALTS OF MAGNESIUM AND CALCIUM IN THE RABBIT.

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PLATE 95.

INTRODUCTION.

On the basis of the hypothesis that magnesium favors inhibition of the various functions of the nervous system, Meltzer and Auer studied extensively in this laboratory the action of magnesium salts upon various animals. In injecting magnesium sulphate subcu- taneously,[1] they found that a certain dose, which varies with the species of animals, is capable of producing profound anesthesia and paralysis from which the animal recovers. For rabbits this dose amounts to about 1.5 gm. of magnesium sulphate ($MgSO_4 + 7 H_2O$) administered in a molecular solution. Larger doses cause the death of the animal, as a rule, by respiratory paralysis. With an effective but non-fatal dose in subcutaneous injections the development of the depressing, inhibitory effect is gradual and fairly slow. When the maximum is reached, the turn for the recovery sets in soon; there is practically no real plateau to the inhibitory curve. The descending limb of this curve—the recovery—is steeper than the ascending one. When a magnesium salt is injected intramuscularly, the inhibitory as well as the fatal effects set in more promptly and with smaller doses.

In the course of their studies, Meltzer and Auer[2] found that calcium, which is chemically closely related to magnesium, is biologically appar-

[1] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905, xiv, 366.

[2] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1908, xxi, 400.

ently the antagonist of the latter.   When calcium is injected intra-
venously, shortly before or immediately after the respiration stops,
into an animal which has received a fatal dose of magnesium, the
animal will recover in less than a minute, provided, of course, that
the circulation is still effective during the calcium injection.   This
biological antagonism is a remarkable fact and can be made the basis
of many problems worth investigating.   So far, at least, it is not
known that between calcium and magnesium salts which have the
same anion a chemical antagonism exists; no precipitation, for in-
stance, occurs *in vitro* when a solution of magnesium chloride is mixed
with a solution of calcium chloride.   Calcium chloride is nevertheless
strikingly antagonistic to magnesium chloride as far as the life of
animals and plants is concerned.

What effect would the deprivation of the animal body of some of
its calcium have upon the behavior of the animal?   There are a
number of acids and salts which precipitate calcium compounds *in
vitro*.   Will the administration of these calcium-precipitating com-
pounds, let us say oxalic acid or oxalates in general, bring out symp-
toms indicating an increase of magnesium action?   By precipitating
calcium within the body a certain amount of unantagonized magne-
sium would be set free.   Would this fact become manifest by the
appearance of inhibitory and paralytic phenomena?   The symptoms
of oxalate poisoning do not speak for it; in general they possess
rather the opposite character: excitation, tremor, and convulsions.
But the amount of magnesium thus set free and the inhibition which
it may be capable of exerting, might under these circumstances be
too small to play a perceptible part, in the presence of the violent
opposite symptoms which are brought out by another exciting factor
of the oxalate.   Could, however, the depressing component of the
calcium-precipitating oxalate be brought out by a simultaneous
administration of a subminimal dose of a magnesium salt?   This
was the problem which we tried to solve experimentally.

While we were at work on this problem, Schütz[3] published a brief
preliminary communication in which he says that the susceptibility
to magnesium injections could be increased occasionally, but not

[3] Schütz, J., *Wien. klin. Wchnschr.*, 1913, xxvi, 745.

constantly by sodium oxalate. A few months later Starkenstein,[4] with whose work we were not familiar until after we had given a preliminary communication of our results,[5] stated in a preliminary report that he found "like Schütz that the addition of oxalates constantly gave a visible increase of the magnesium narcosis."

We shall describe briefly our experiments bearing upon the problem under discussion and the conclusions to which they point.

EXPERIMENTAL PART.

We experimented exclusively on rabbits. Magnesium sulphate in M solution (MgSO$_4$ + 7 H$_2$O) and sodium oxalate in 3 per cent solution[6] were injected separately and practically simultaneously, either into the lumbar muscles on opposite sides of the spine, or subcutaneously into each flank, the injection being usually followed by brief massage. All doses were estimated and are here reported in gm. of the salt per kilo of body weight. Most of the experiments were performed on a series of three animals, two serving as controls and receiving subtoxic doses of either magnesium sulphate or sodium oxalate alone. The experimental animal received the same dose of both salts.

Intramuscular Injections.

An abbreviated typical protocol follows.

Experiment I.

Rabbit A.—Oct. 2, 1913. Magnesium sulphate alone. Grey female. Weight 1,550 gm.

11.10 Right lumbar muscles: magnesium sulphate M, 4.3 cc. = 0.7 gm. per kilo of body weight.

11.23. Lying down, head up, breathing rapidly.

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[4] Starkenstein, E., *Wien. klin. Wchnschr.*, 1913, xxvi, 1235.

[5] Gates, F. L., and Meltzer, S. J., *Zentralbl. f. Physiol.*, 1913–14, xxvii, 1169. Starkenstein, E., *Zentralbl. f. Physiol.*, 1914, xxviii, 63; *Arch. f. exper. Path. u. Pharm.*, 1914, lxxvii, 45.

[6] Merck's reagent, "Sörensen's oxalate." Impure oxalates are not soluble to 3 per cent.

11.32.   Can be placed on side.

11.34.   Moves eyes and head when approached.

11.40.   When tail is pressed, raises head and turns on belly. Moves head and looks around.

11.57.   Hops when tail is pressed.

12.13.   Hops around voluntarily, watching other rabbits. Remains well.

Rabbit B.—Oct. 2, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,720 gm.

11.13.   Right lumbar muscles: magnesium sulphate M, 4.8 cc. = 0.7 gm. per kilo. Left lumbar muscles: sodium oxalate 3 per cent, 5.6 cc. = 0.10 gm. per kilo.

11.24.   Lying prone, with head on floor. When pushed over on side, lies passive. Respiration full; 17 in ¼ min. No response to pressure on tail; lid reflex good.

11.38.   Same position. Lid reflex hardly perceptible.

12.01.   Respiration slow and deep; 13 in ¼ min.

12.12.   No response to stimuli, no lid reflex.

12.37.   Turns over and lies on belly. Head sinks to floor and is raised at intervals. No response to pressure on tail.

1.08.   Crouching in a corner. Recovers.

Rabbit C.—Oct. 2, 1913. Sodium oxalate alone. Grey male. Weight 1,480 gm.

11.21.   Left lumbar muscles: sodium oxalate 3 per cent, 4.88 cc. = 0.1 gm. per kilo of body weight.

11.28.   Sitting up, alert, changes position frequently. Starts suddenly without apparent cause.

11.43.   Active, hops around, laps water, licks site of injection.

12.14.   Has been behaving normally. Does not remain in one position long. No further effects noted.

The contrast in the behavior of the controls and the experimental animal is striking. The control animals were but little affected: this dose of sodium oxalate produced only trivial symptoms in Rabbit C, and the magnesium animal, Rabbit A, while weak and stupid, was at no time paralyzed or anesthetic. The third rabbit, however, ten minutes after the injections, was deeply anesthetized and remained passive and insensible for an hour. Two points are of particular interest: (1) In spite of the depth and long duration of the narcosis the respiration continued of good volume and rate and the animal was at no time in danger. (2) The animal regained power of voluntary movement before the return of sensibility to painful stimuli. A series of experiments with similar doses is given in Table I.

TABLE I.

Magnesium Sulphate and Sodium Oxalate, Intramuscularly.

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	*gm.*	*min.*		
6	0.7	0.10	89	0	.
6	0.7	—	10	0	Only two out of six relaxed at all.
4	—	0.10	0	0	Visible effect questionable.

Subcutaneous Injections.

When the injections were made subcutaneously, somewhat larger doses had to be employed. A typical protocol of an experiment follows.

Experiment II.

Rabbit A.—Oct. 9, 1913. Magnesium sulphate alone. Slate colored female. Weight 2,030 gm.

10.16. Left flank, subcutaneously: magnesium sulphate M, 6.5 cc. = 0.8 gm. per kilo. Massage for 20 seconds.

10.31. Lying down, head and ears erect, breathing rapidly.

10.47. When disturbed hops away clumsily.

11.15. Crouching quietly, head up, ears flat on back. Respiration good.

11.50. Raises head to sniff at nearby objects.

12.20. Sitting up, washing paws. Remains well.

Rabbit B.—Oct. 9, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,755 gm.

10.18. Right flank, subcutaneously: magnesium sulphate M, 5.6 = 0.8 gm. per kilo. Left flank, subcutaneously: sodium oxalate 3 per cent, 8.75 cc. = 0.15 gm. per kilo. Both sides massaged for 20 seconds.

10.53. Sitting up naturally.

11.04. Lying with chin on floor. Respiration slower and deep; 21 in ¼ min.

11.13. Placed passively on side without a struggle. Respiration 18 in ¼ min.

11.37. No response to pressing tail. Respiration shallow.

12.50. Trace of lid reflex. No response to pressing tail. Respiration of fair depth; 14 in ¼ min.

2.35. No lid reflex. No response to pressing tail.

3.52. Animal lying as before. No response to stimuli. Breathing entirely abdominal, of fair depth; 14 in ¼ min.

4.28. Does not resist handling. Voluntarily moves head, tail, and legs slightly. Observation discontinued.

Oct. 10, 1913. 9.15. Sitting up in cage. Rather quiet.

Rabbit C.—Oct. 9, 1913. Sodium oxalate alone. Grey female. Weight 1,510 gm.

10.22. Left flank, subcutaneously: sodium oxalate 3 per cent, 7.5 cc. = 0.15 gm. per kilo. Massage for 20 seconds.

10.43. Hops around licking the floor and sniffing at objects.

10.53. Sitting up, behaving normally.

11.14. Hops off actively when approached. No effects noted from injection.

Here again neither the oxalate nor the magnesium alone was effective. Together they produced a profound depression with a period of anesthesia and paralysis lasting more than four hours, followed by a gradual complete recovery.

Table II summarizes experiments with subcutaneous injections.

TABLE II.

Magnesium Sulphate and Sodium Oxalate, Subcutaneously.

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	*gm.*	*min.*		
8	0.8	0.15	123 +++	1	In four animals anesthesia extended into the night following. One died next day without recovery.
8	0.8	—	0	0	Practically no effect. Drowsiness in four cases.
8	—	0.15	0	0	No effects observable.

The cited protocols and the two tables illustrate the results obtained in these series of experiments. With the exception of two failures at the beginning, before the proper relation of dosage was determined, the experimental animal in every instance was definitely more deeply affected than the controls. The differences between the various experiments were only of degree, and depended upon the relation of the dose employed and the mode of administration, whether subcutaneous or intramuscular. With proper dose the

contrasts were striking and constant; while the controls were hardly visibly affected, the experimental animals were deeply anesthetized and paralyzed, the character of this inhibition being in general similar to that caused by large effective doses of magnesium alone.

In the following particulars the depression of the animals which received sodium oxalate and magnesium sulphate seemed to differ from that of animals which received magnesium alone. (1) The period of anesthesia and paralysis is a fairly long one, especially after subcutaneous injections, when the state of inhibition may last even 4 hours and longer; whereas after an effective sublethal dose of magnesium alone the entire state of depression is of a comparatively short duration. (2) In animals which receive oxalate and magnesium the deepest stage of anesthesia and paralysis tends to become stationary and is of long duration—the inhibitory curve has a long plateau—and the recovery takes place gradually; whereas with magnesium alone the inhibitory curve has hardly any plateau, and the animal after reaching the acme of anesthesia and paralysis either recovers quite rapidly or the depression leads to death by respiratory paralysis.

The increase of depression following the injection of subminimal doses of sodium oxalate and magnesium sulphate which was definitely established in these experiments cannot be considered simply as a summation of two similar effects. The symptoms brought on by oxalates are entirely dissimilar to those of magnesium inhibition. In our experiments the symptoms which follow the injections of sodium oxalate in toxic doses exhibit the character of excitation; anxiety, restlessness, hypersensitiveness, and tonic and clonic convulsions, which finally lead up to asphyxia and to a fatal termination. In such subminimal doses as we have employed, the toxic symptoms, if there were any, consisted at most in excitation and increased alertness; but there was never any manifest depression. It seems, therefore, that the strikingly depressing effect which the addition of a practically non-toxic dose of sodium oxalate to a subminimal dose of magnesium produces, must be ascribed to the ability of the oxalate to precipitate calcium from the body fluids and thus eliminate an element which biologically is antagonistic to magnesium.

The Action upon the Motor Nerve Endings.

Among the general effects of magnesium salts their depressing action upon the motor nerve endings stands out prominently. In minimal effective doses these salts reduce and in larger doses they completely abolish the conductivity of the nerve endings. In a series of experiments we have studied directly the combined action of sodium oxalate and magnesium sulphate upon this intermediary link between nerve and muscle. The sciatic nerve was cut under ether, the animal permitted to recover completely, and then the motor reactions of foot and toes to faradic stimulations of the peripheral end of the sciatic nerve were studied under the influence of the salts under discussion. Seventeen experiments were made upon rabbits. In fifteen there were two rabbits to each experiment, one an experimental animal and one a control. The experimental animals received subcutaneous injections of 0.6 to 0.8 gm. of magnesium sulphate in one side and 0.15 to 0.2 gm. of sodium oxalate. The fifteen control animals received injections of 0.6 to 0.8 gm. of magnesium sulphate alone. Two rabbits received injections of 0.15 and 0.2 gm. of sodium oxalate alone. For faradic stimulations a Porter induction coil, armed with one Daniell cell, was used. The cut sciatic nerve was stimulated before and at various intervals after the injection of the salt solutions, and the degree of the reactions to the various strengths of stimuli was noted. The results obtained in the experimental and control animals were compared and brought into relation with the general condition of the respective animals.

In both the sodium oxalate animals stimulation of the sciatic nerve before and at various times after the injection gave prompt reactions; strong tetanic flexion of the foot and abduction of the toes.

In eight of the magnesium controls stimulation of the sciatic nerve gave normal responses at the various periods after the injection. In the seven other controls there were slight degrees of reduction in the response to the stimulations; the reaction was less prompt, the extent of the contractions was lessened, or the distance of the secondary coil, in order to be effective, had to be shortened.

Of the experimental animals, in thirteen the conductivity of the peripheral nerve endings was definitely more deeply affected than in

their controls. In some cases the conductivity was so depressed that at the time when the general narcosis was at its height no response could be obtained from the stimulation of the sciatic nerve even with a 40 mm. coil distance. In two of the experimental animals the reduction in the response to stimulation of the sciatic nerve was not greater than that of their controls, although the general signs of anesthesia in the experimental animal were quite deep.

The depressing effect upon the motor nerve endings never outlasted the central effects, while there were cases in which the loss of sensation still continued after the motility seemed to be normal again.

The Antagonistic Action of Calcium.

Calcium, as stated in the introduction, is biologically antagonistic to magnesium, and our present experimental results led us to the conclusion that the increase of the depressive action of subminimal doses of magnesium by the addition of a subtoxic dose of sodium oxalate was due to the calcium-precipitating property of this salt. On the other hand, we found that the anesthesia and paralysis produced by a combination of subminimal doses of the two salts was of much longer duration than the same condition produced by an effective dose of magnesium sulphate alone. The question presented itself: Would calcium cause a recovery from the profound long-lasting state of depression caused by the combined action of the two salts, and especially would the recovery be as prompt and as rapid as in cases of magnesium anesthesia? We made a large number of experiments, but our results may be presented in the following single sentence: The antagonistic action of calcium is just as striking and prompt in the prolonged anesthesia brought about by the combination of oxalate and magnesium as it is in the anesthesia produced by magnesium alone. The following protocol is typical for all experiments in this series, and the photographs (Figs. 1 and 2) taken of this experiment are a good illustration of the results.

Experiment III.

Rabbit I.—Mar. 9, 1914. Magnesium sulphate alone. Grey and white male. Weight 1,860 gm.

1.58. Right back, subcutaneously: magnesium sulphate м, 5.9 cc. = 0.8 gm. per kilo. Massage for 1 min.

2.16. Sits quietly in corner of box, or lies down.

2.23. Crouching on forepaws, head and ears up. Respiration fair volume, slow; 19 in ¼ min.

2.32. Lying at full length, head and ears up. Backs up into sitting posture; rather heavy and quiet.

2.47. Crouching quietly in corner of box. Respiration full volume; 14 in ¼ min.

3.07. Photographed (Fig. 1).

3.16. Photographed (Fig. 2).

3.45. Behaving normally and has shown no further effects. Remains well.

Rabbit II.—Mar. 9, 1914. Magnesium sulphate and sodium oxalate. Black and white female. Weight 1,540 gm.

2.01. Right back, subcutaneously: magnesium sulphate M, 4.9 cc. = 0.8 gm. per kilo.

2.03. Left back, subcutaneously: sodium oxalate 3 per cent, 7.7 cc. = 0.15 gm. per kilo. Massage both sides for 1 min.

2.10. Has defecated. Respiration rapid and rather deep. Restless, changes position often.

2.13. Hind legs dragged a little in walking.

2.21. Crouching, head up, ears back, breathing rapidly; 68 in ¼ min.

2.35. Lying full length, eyes half closed, ears back, chin on floor. Flanks relaxed and bulging. Respiration 50 in ¼ min.

2.49. Lying partly on side, relaxed, head flat on floor. Mere trace of lid reflex. Moves head slightly when tail is touched.

3.05. Placed passively on back, feet in air. Remains there relaxed.

3.07. Photographed with controls (Fig. 1).

3.15. Same condition. Given 8 cc. calcium chloride 0.125 M through left ear vein. Respiration deepens during injection, and before it is completed animal turns over and sits up.

3.16. Photograph taken within 1 minute of injection (Fig. 2).

3.45. Crouching quietly. Hair erect. Hops off actively when disturbed. Then sits up with head and ears up. Remains well.

Rabbit III.—Mar. 9, 1914. Sodium oxalate alone. White female. Weight 1,620 gm.

2.06. Left back, subcutaneously: sodium oxalate 3 per cent, 8.1 cc. = 0.15 gm. per kilo. Massage for 1 min.

2.11. Hind legs dragged a little at times. Rather restless.

2.30. Sitting up or hopping around naturally. Head and ears up. Not restless or anxious. Respiration 42 in ¼ min.

2.48. Behaving normally. Sitting up, quiet. Respiration 35 in ¼ min.

3.07. Photographed (Fig. 1).

3.16. Photographed (Fig. 2).

3.45. Has shown no further effects.

Figs. 1 and 2 illustrate, in the first place, the anesthesia and paralysis produced by the combination of subminimal doses of magnesium and oxalate. They show, further, in a striking way, the antagonistic action of intravenous injection of calcium; it is in all respects similar to the action of calcium in anesthesia by magnesium alone. The respiration becomes deeper and more rapid immediately after beginning the injection, and the return of muscle tone and motor activity can be felt under the hand. Within a minute after the beginning of the injection, often indeed before all of the 8 or 10 cc. of solution is given, the animal draws up its legs, raises its head, turns over and scrambles into a sitting posture, and becomes alert and inquisitive. After an interval the rabbit may gradually sink back into narcosis, and can be restored again by calcium. Occasionally, if too much magnesium and oxalate have been given, a third injection may still be needed and given with success. However, under such circumstances, repeated injections of calcium might finally prove fatal to the animal.

The experiments, showing the depressing effect of magnesium and the antagonistic action of calcium to this depression, are, as we had occasion to learn, frequently demonstrated in many European Universities in lectures on pharmacology or physiology. When magnesium alone is used, the period of the greatest depression is of short duration and the demonstration may either be unconvincing, when the animal is not yet sufficiently narcotized, or it may be a failure, when the calcium injected is administered too late. The anesthesia and paralysis brought about by a combination of sodium oxalate and magnesium sulphate is, as we have seen above, of comparatively long duration. It is therefore a more appropriate method for purposes of demonstration. The animal may receive its double injection 40 to 50 minutes before the time set for the demonstration. If the proper doses are given and the proper procedure is followed out, there is no danger that the animal will not be in deep anesthesia, or that it will die too soon, before the antagonistic effect of the calcium can be shown.

SUMMARY.

The foregoing experiments establish firmly the following facts.

Subcutaneous or intramuscular injections of sodium oxalate in sub-toxic doses, when administered to an animal which received a sub-minimal dose of magnesium sulphate, produce profound anesthesia and paralysis of long duration, although the usual effects of sodium oxalate alone are of a stimulating character. This fact is, in general, in harmony with the results reported by Starkenstein who, however, seems to have used the combination of the two salts in one solution; namely, that of magnesium oxalate.

The combined injections of subminimal doses of sodium oxalate and magnesium sulphate produce a strong reduction, or even, at times, a complete abolition of the conductivity of the motor nerve endings.

An intravenous injection of calcium salts brings on a recovery from the profound and prolonged effects of the combined action of sodium oxalate and magnesium sulphate, which is as prompt as is observed in experiments in which effective doses of magnesium alone were given. This fact is the more noteworthy, since depressions of long duration produced by prolonged continuous injections of magnesium solutions alone do not respond very promptly and effectively to calcium injections.

As will be recalled, the starting point for our investigation was the hypothesis that substances which are capable of precipitating calcium—a biological antagonist of magnesium—ought to be capable of increasing the depressive effect of magnesium. Our experiments proved that this assumption was correct. This would seem, therefore, to justify the interpretation that the augmenting action of sodium oxalate has its cause in the ability of the latter to precipitate calcium and thus increase within the body the amount of unantagonized magnesium. However, we wish to state expressly that this view is, for the present, still no more than a hypothesis and does not exclude other possible interpretations of our facts. As we pointed out it speaks against this hypothesis that oxalates do not produce phenomena of depression; the toxic symptoms produced by oxalates exhibit distinctly signs of increased and not of decreased irritability.

FIG. 1. Rabbit III. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Alert, ears erect. (Caught by instantaneous exposure.) Rabbit II. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Magnesium sulphate M, 0.8 gm. per kilo. Deeply anesthetized and quite relaxed. Rabbit I. Magnesium sulphate M, 0.8 gm. per kilo. Crouches quietly as placed. Ears back.

FIG. 2. Rabbits III and I as before. Rabbit II within a minute has received 8 cc. of calcium chloride 0.125 M into the marginal ear vein (note clip). Alert and sensitive; right paw blurred from movement.

III   II   I

FIG. 1.

III   II   I
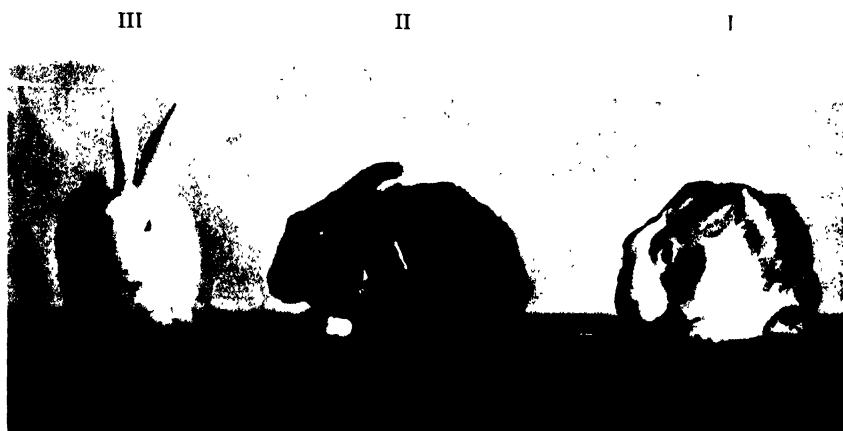
FIG. 2.

THE ABSORPTION OF ADRENALIN AFTER INTRATRACHEAL INJECTION.*

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In order to subject a living organism to the systemic action of any soluble substance, it is obvious that the substance must first reach the circulating fluids of this organism; from the lymph and blood streams the drug then may pass into the tissues and exert its effect. The main routes available for bringing any substance into contact with the tissues are as follows: (1) by introduction into the gastro-intestinal canal; (2) by subcutaneous, intramuscular, intravenous, or intraspinal injection; (3) by inunction through the skin; and (4) through the respiratory tract.

Of these routes the first two are most frequently employed in human therapeutics and in the laboratory. Inunction is used only exceptionally at the present time, and practically the only drug administered through the skin for its systemic action is mercury.

The respiratory route also is not utilized to any extent, except for purely local effects, when it is desired to incorporate a drug. Syphilis was occasionally treated by allowing the patient to inhale the sublimated metal, but this method was never extensively employed because of the impossibility of judging the dose. Local affections of the respiratory passages, for example, laryngitis, bronchitis, and bronchiectasis, are treated by allowing the patient to inhale the vapors of boiling water to which various substances (creosote, eucalyptol, opium preparations, etc.) have been added. Experimentally the respiratory passages are practically not utilized when a drug is to be administered for its general action.

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* A preliminary report was published in the Proceedings of the Pharmacological Society (*Jour. Pharm. and Exper. Therap.*, 1914–15, vi, 608).

Among the relatively few experimenters who have used the intratracheal method for this purpose we may mention Külbs,[1] who injected rabbits repeatedly for a number of days with adrenalin, introducing the hypodermic needle into the trachea through the skin of the neck. He observed cough after the injection, and twice an animal died a few minutes after the injection, from pulmonary edema. In those animals which survived the injections, which were given every day or every other day for 22 to 78 days, the total amount being 3.4 to 14 cc. of adrenalin, he found the same macroscopic and microscopic alterations, though of smaller extent, which he obtained after intravenous injection of adrenalin.

Ephraim[2] in a series of papers reports the results of endobronchial treatment of chronic bronchitis and asthma in the human subject. Various drugs, including adrenalin, were introduced as a fine spray into the bronchi, often through a bronchoscope. The effects obtained were chiefly local; even after the bronchial administration of 1 mg. of adrenalin by means of a nebulizer, he obtained no definite rise of blood pressure. In a dog, however, the same method yielded a powerful rise of blood pressure when a large amount, 2 mg., was used.

The failure of Ephraim to obtain a rise of pressure after the administration of 1 mg. of adrenalin endobronchially in the human subject is perhaps attributable to the method. Ephraim himself observed in rabbits that the spray of a colored solution from a nebulizer, even when introduced into the trachea, did not reach the bronchi, the vapor being precipitated near the point of application. In the same way the endobronchial spray of adrenalin was possibly precipitated in the larger bronchi so that only negligible amounts reached the alveolar region where, as we shall show, the absorption is best.

That even colloids can be absorbed with rapidity when injected intratracheally is shown by Ishioka.[3] Ishioka sensitized guinea pigs by the subcutaneous injection of human serum and after 12 to 15 days injected 0.05 to 0.1 cc. of human serum into the trachea of these animals with the object of producing an anaphylactic pneumonia. He observed the milder anaphylactic symptoms in the majority of his experiments, but in two instances acute death with the typical lung picture resulted.

The absorptive capacity of the supratracheal respiratory passages does not concern us here; references concerning the effect of inhaled substances will be found in the papers by Ephraim.

Our own experiments will show that the intratracheal injections of adrenalin are rapidly absorbed even under disadvantageous conditions and exert a systemic effect, and also that this method may be of value therapeutically when a rapid action on the heart is desired.

[1] Külbs, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 150.

[2] Ephraim, A., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 2079; 1912, xxxviii, 1453.

[3] Ishioka, S., *Deutsch. Arch. f. klin. Med.*, 1912, cvii, 500.

Methods.

The research was carried out entirely with rabbits, and the chief test substance employed was adrenalin. Adrenalin was chosen because its absorption could be readily detected by recording the blood pressure, and the character and degree of the resultant rise would give some indication of the speed and amount of absorption.

The animals were anesthetized by ether for the operative interferences. These were the insertion of a cannula into the right carotid artery; transection of the trachea with introduction and ligation of a wide glass tube about 2 cm. long into the distal stump so that the respiratory path was free and injection into the trachea easy; in some rabbits the tracheal cannula was inserted as near the thorax as possible and the lower end of the proximal trachea then ligated, thus converting the cervical trachea into a sac from which the absorption of adrenalin could be tested; in a number of experiments both vagi were sectioned in the neck.

The blood pressure was written by a mercury manometer connected with the artery by tubing filled with a half saturated solution of sodium sulphate.

The adrenalin used was generally the commercial solution in 0.1 per cent strength, preserved with chloretone. In some experiments, however, a solution was made from the commercial powder preparation, and this was used with or without the addition of chloretone.

The dose injected varied from 0.15 to 0.03 cc. per kilo of body weight. The maximum dose quoted was occasionally greatly exceeded when adrenalin was injected intramuscularly.

The solutions were injected into the respiratory passages in several ways. Usually the required amount was injected in 4 to 6 seconds from a Record tuberculin syringe into the tracheal cannula. In a number of experiments a filiform catheter was introduced into a bronchus and the solution then driven in by a gentle blast of air. A few times the solution was injected directly into the lung tissue by passing the hypodermic needle through the walls of the chest.

In a majority of the experiments doses of adrenalin were injected repeatedly, not only into the trachea but also into the erector spinæ muscles of the back.

The rabbits were always placed on an electric warming pad to reduce or prevent the loss of body heat.

EXPERIMENTAL RESULTS.

Twenty-three experiments were carried out in the adrenalin series; in ten of these tests both vagi were cut previous to the injection of the drug. The course of the experiments and the characteristic effects will be illustrated by a few typical protocols arranged in tabular form.

All rabbits tabulated were tracheotomized under light ether anesthesia; there was no insufflation of air except in No. 3 (Table I); vagi

were intact, except in No. 8 (Table IV); all injections into the trachea were made with a syringe through the tracheal tube.

TABLE I.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	sec.	mm.	min.	min.
3		1	Catheter into bronchus.	0.23	12	64 (104–168)	8	
		2	Trachea.	0.23	—	45 (90–135)	9	9
	Gray ♀	3	" "	0.23	6	25 (107–132)	9	10
Vagi intact.	2,210 gm.	4	Erector spinæ muscle.	0.23	No rise in 4 min.			10
		5	Erector spinæ muscle.	0.46	" " " 4 . "			4
		6	Trachea.	0.23	7	24 (96–120)	18	4

Killed by medullary puncture after experiment.   Lungs showed only a moderate degree of pulmonary edema.

6		1	Tracheal sac.	0.25	52 ·	16 (91–107)	15	
		2	Trachea. ·	0.25	12	60 (100–160)	—	17
	Gray ♂	3	Tracheal sac.	0.25	No rise in 6 min.			16
Vagi intact.	2,100 gm.	4	Trachea.	0.25	3	40 (62–102)	10	8
		5	Erector spinæ muscle.	0.5	No rise in 7 min.			10
		6	Trachea.	0.25	4	26 (80–106)	7	10

Killed later by medullary puncture.   The lower lobes of both lungs showed well marked pulmonary edema; slight in upper lobes. ·

TABLE II.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	sec.	mm.	min.	min.
7		1	Trachea.	0.12	2	50 (100–150)	6	
		2	"	0.12	4	39 (88–127)	12	9
	Gray ♂	3	Erector spinæ muscle (left).	0.6	3	43 (99–142)	20	13
	1,610 gm.	4	Trachea.	0.12	8	21 (98–119)	10	21
Vagi intact.		5	"	0.12	5	25 (95–120)	12	15
		6	Erector spinæ muscle (right).	0.28	No rise in 8 min.			14
		7	Trachea.	0.12	3	24 (92–116)	21	8
		8	"	0.24	10	10 (102–112)	5	20

Killed later by medullary puncture. Lungs collapsed well and showed only a moderate degree of pulmonary edema.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
8		1	Trachea.	0.21	6	64 (114–178)	15	
Vagi cut.	White ♀	2	"	0.21	10	30 (110–140)	6	18
	2,365 gm.	3	"	0.21	8	16 (116–132)	9	8
.		4	Erector spinæ muscle.	0.42	No rise in 4 min.			15

Pink fluid poured from trachea 13 min. after first dose. About 5 min. after last injection, blood pressure fell abruptly, convulsions, death. Lungs showed marked pulmonary edema.

TABLE III.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	*sec.*	*mm.*	*min.*	*min.*
4		1	Erector spinæ muscle (left).	0.23	14	34 (106–140)	4	
		2	Jugular vein.	0.23	At once.	43 (107–150)	10	9
Vagi intact.	Gray 2,160 gm.	3	Trachea.	0.23	8	20 (110–130)	10	9
		4	Erector spinæ muscle (right).	0.23	No rise in 2 min.			11
		5	Trachea.	0.23	10	10 (100–110)	3	2
		6	Erector spinæ muscles.	0.23	No rise.			

After last injection foam and pink fluid poured from trachea (marked pulmonary edema).

A study of these typical protocols as given in Tables I, II, and III shows with clearness all the points we wish to emphasize, and they will now be considered in detail.

Latent Period.—The latent period elapsing between injection and the onset of the blood pressure rise varied between 2 and 38 seconds, but usually was less than 10 seconds; the general average of all first injections was 13 seconds. Repeated injections exerted no uniform effect on the length of the latent period. Thus in Experiment 7 the first dose of 0.12 cc. of adrenalin per kilo injected into the trachea surely reached a physiologically effective concentration in the blood within 2 seconds, for after that interval the blood pressure began to rise. The seventh injection of the same dose in the same place, 80 minutes later, exerted a blood pressure effect after a latent period of only 3 seconds, in spite of the fact that the animal had received intratracheally and intramuscularly in the interval between the two injections mentioned 1.24 cc. of adrenalin per kilo, divided into five doses. A still more striking example of the speed of absorption is

given in Experiment 8. In this experiment the vagi had been cut before administering the adrenalin, and the first dose of 0.21 cc. per kilo into the trachea caused a pulmonary edema of such an extent that foam and pinkish fluid poured from the tracheal cannula. Nevertheless, a repetition of the same dose in the same place a few minutes later exerted a good blood pressure effect after a latent period of only 10 seconds. In this instance the area available for absorption was undoubtedly reduced considerably by the pulmonary edema, yet the speed of absorption was still rapid. All the tabulated protocols show a similar rapid absorption after intratracheal injections.

The latent period or speed of absorption was not apparently affected by the amount of adrenalin injected; for example, in Experiment 3 the intrabronchial injection of 0.23 cc. per kilo by means of a catheter showed a latent period of 12 seconds before the blood pressure began to rise. In Experiment 7, on the other hand, a much smaller dose per kilo, 0.12 cc., injected into the trachea gave a blood pressure effect after a latent period of only 2 seconds.

Quantitative Absorption.—The quantitative absorption cannot be definitely established, yet the blood pressure rises obtained after the various injections furnish some indication of the amount of adrenalin absorbed. Examination of the absolute values of the blood pressure rises in mm. of mercury obtained after successive injections of the same dose of adrenalin in the same place, the lung passages for example, shows that the blood pressure effect in general decreases with the number of injections. Thus in Experiment 7 five doses of 0.12 cc. of adrenalin per kilo were injected into the trachea, the time intervals varying between 9 and 34 minutes if the interpolated intramuscular injections are included. The blood pressure rises after these five tracheal injections were 50, 39, 21, 25, and 24 mm. In Experiment 8 the successive decrease of effect is more pronounced. In this experiment 0.21 cc. of adrenalin per kilo into the trachea at intervals of 8 to 18 minutes gave the following rises of blood pressure: 64 ,30, and 16 mm. In this animal, however, the vagi had been sectioned previous to the first injection of adrenalin, and after the first dose a marked pulmonary edema developed during which fluid poured from the tracheal cannula. The presence of so much fluid decreased the absorptive area available, and in addition diluted the adrenalin with a colloidal

solution, rendering absorption still slower, yet in spite of this the speed of absorption was not appreciably delayed, the latent period being 10 and 8 seconds, but the amount absorbed, as judged by the pressure rises, showed practically 50 per cent decreases.

This decrease in effectiveness in causing a blood pressure rise shown by successive tracheal injections is in accord with the observations of Meltzer and Auer[4] that adrenalin diminishes absorption from the tissues. These authors demonstrated, among other facts, that the intravenous injection of adrenalin delays the absorption of strychnine or fluorescein from the subcutaneous tissue, and also that repeated intramuscular injections give a decreasing blood pressure effect, due to diminished absorption.

It will be noticed on examining the tables that all injections of adrenalin into the trachea gave some blood pressure effect; in other words, that some absorption took place even under unfavorable circumstances. A comparison was therefore made between the absorptive capacity of the lung and that of the erector spinæ muscles. The erector spinæ muscle was chosen because this thick mass of muscle is composed of fine fibers not separated into coarse fasciculi, like the glutei for example, and in addition is surrounded by a dense fascia which exerts pressure upon the injected substance, thus facilitating absorption. Injection into this muscle has been shown by Meltzer and Auer[4] to be practically equivalent to an intravenous injection.

The experimental test gave important results. It clearly appeared that the injection of several doses of adrenalin into the lung passages reduced the absorption from the erector spinæ muscles to such a degree that even double the intratracheal dose given intramuscularly did not enter the circulation in sufficient amount to affect the blood pressure; nevertheless, another injection of the original dose of adrenalin into the lungs promptly entered the circulation in sufficient concentration and amount to cause a blood pressure rise (see Experiments 3, 6, 7, and 4). This fact appears with especial clearness in Experiment 3. In this test the rabbit received three doses each of

[4] Meltzer, S. J., and Auer, J., *Tr. Assn. Am. Phys.*, 1904, xix, 207; *Jour. Exper. Med.*, 1905, vii, 59. Auer and Meltzer, *ibid.*, 1911, xiii, 328.

0.23 cc. of adrenalin per kilo into the lung, the time consumed being about 29 minutes. Each injection gave a rise of blood pressure (64, 45, and 25 mm.). Then the same dose was injected intramuscularly, but no blood pressure rise occurred in 4 minutes. The dose was then doubled and 0.46 cc. per kilo was injected into the other erector spinæ muscle, but again no blood pressure rise followed. 4 minutes later the original dose of 0.23 cc. was injected into the lung passages and after a latent period of 7 seconds the blood pressure began to rise and reached an absolute value of 24 mm. of mercury.

The failure to obtain a blood pressure effect from an intramuscular injection of adrenalin under the conditions mentioned is, however, not absolute. If the dose administered intramuscularly is increased sufficiently, enough adrenalin will be absorbed to cause a blood pressure rise. This is illustrated in Experiment 7. Two lung injections of 0.12 cc. of adrenalin each had been injected in about 22 minutes. Then five times the dose (0.6 cc. per kilo) was injected intramuscularly; after a latent period of 3 seconds the blood pressure rose 43 mm. That there was, nevertheless, a definitely diminished absorption from this large intramuscular injection is shown by the fact that the rise of blood pressure, 43 mm., is even less than that caused by the first lung injection of only one-fifth the dose, which latter raised the pressure 50 mm. Subsequent injections of adrenalin into the lungs all gave rises of blood pressure, but an interpolated intramuscular injection of double the pulmonary dose gave no blood pressure effect. The amount absorbed from the muscles had fallen below the level of a physiologically effective dose.

In some experiments the conditions were still further varied by preceding the lung injections of adrenalin by intramuscular and intravenous injections of the same substance. Experiment 4 is one of this type. The adrenalin dose was always 0.23 cc. per kilo. After an intramuscular and an intravenous injection, absorption of adrenalin from the lung was by no means prevented, the blood pressure rising 20 mm. after a latent period of 8 seconds. A subsequent intramuscular dose, however, produced no blood pressure rise within 2 minutes. Another lung injection even now caused a definite rise of pressure.

These experiments definitely show that, under the conditions

mentioned, absorption from the lungs occurs with doses of adrenalin which are ineffective when injected intramuscularly.

There was no definite relationship to be observed between the amounts of adrenalin injected into the lungs and the resultant blood pressure rise. The same dose per kilo often produced widely different rises in different animals, and smaller doses often caused greater elevations of pressure than larger ones. Table IV illustrates this and demonstrates in addition that the amount of adrenalin injected played no part.

TABLE IV.

		gm.				*mm.*
No. 5	Vagi intact.	1,650	0.3 cc. per kilo (0.5 cc.)	4 sec. latent period.		33 rise (107– 140)
" 7	" "	1,610	0.12 cc. per kilo (0.2 cc.)	2 " " "		50 rise (100– 150)
" 8	" cut.	1,980	0.25 cc. per kilo (0.5 cc.)	18 " " "		30 rise (114– 144)
" 13	" "	1,790	0.25 cc. per kilo (0.4 cc.)	18 " " "		82 rise (116– 198)

Blood Pressure.—The duration of the blood pressure elevation was usually less than 10 minutes; if only the effects of the first tracheal injections are considered the average is 6 minutes. Subsequent injections, however, often showed a definitely longer duration of the elevation. An illustration of this effect will be found in Experiment 7.

The character of the rise varied somewhat; it was usually more or less abrupt, the maximum being reached within 30 seconds. In other instances the maximum elevation was reached in about 1 minute. The abruptness of the rise seemed to bear some relation to the number of preceding injections, the slope becoming less steep with succeeding doses. This, however, was by no means true of all experiments, for in some all intratracheal injections gave sharp rises of pressure. Vagus pulses were often observed, but their occurrence was not as frequent as when the adrenalin is administered intravenously or intramuscularly.

In a number of experiments abrupt and profound drops of blood pressure were noted. These occurred without any warning during the maximum pressure elevation, the pressure falling within a few

seconds to 20 mm. and even less. These drops lasted from 30 to 160 seconds, and were not necessarily fatal. In some instances a number of these profound drops occurred, recovery of the blood pressure taking place spontaneously after a series of convulsions. When these drops occurred, they were always associated with more or less pronounced signs of pulmonary edema. The significance of this phenomenon will be discussed in a later paper.

Site of Absorption.—In order to obtain some information regarding the site of absorption of the injected drug, a few experiments were made with intratracheal injections of India ink or suspensions of lampblack in an extremely dilute gum arabic solution. Doses of 0.3 cc. per kilo of body weight were injected slowly by syringe into the tracheal cannula. 10 seconds after the injection the medulla was destroyed by puncture, and the lungs and trachea were immediately excised and examined. The three experiments carried out gave concordant evidence: the posterior and diaphragmatic surfaces of the lower left lobe always showed a large number of discrete and confluent, irregular black spots varying from about 2 to 5 mm. in diameter. The surfaces of the other lobes showed only a few or no spots, and these were confined largely to the posterior surfaces near the hilus of the right lower and right middle lobes.

On sectioning the lungs through the trachea and bronchi, the larger part of the left lower lobe was found to be a black mass containing foam and some fluid. The right lower and right middle lobes near the hilus also contained an amount of pigment which was greater than would be expected from the surface indications. The upper lobes and the median lappet showed a few spots in the body of these divisions.

The sections of the lung containing the pigment were larger and fuller than those free from it; moreover, they contained more fluid. The distension was greater than the amount of fluid present explained, and apparently was at least partly caused by a mechanical plugging of the bronchioles and infundibular ducts by the pigment. The amount of fluid present in the tissues seemed greater than the amount injected (0.3 cc.), so that perhaps some degree of pulmonary edema also developed.

These experiments with the tracheal injection of pigment suspen-

sions thus indicate that a certain amount penetrates to the alveoli, chiefly of the left lower lobe, within less than 1 minute, and that absorption in all likelihood takes place there.

It might be thought that some absorption could take place from the mucosa of the trachea and the bronchi, and such absorption indeed does take place, at least as far as the tracheal mucosa is concerned. This absorption from the tracheal mucosa is, however, quite slow and the blood pressure rise obtained sets in very slowly. We tested the absorptive power of the tracheal mucosa in the following way. The tracheal cannula was inserted as low as possible in the neck; the upper section of the trachea was then ligated near the cannula, converting it into a sac. Injections of adrenalin were then made into this sac and the blood pressure effect was noted. In the two experiments made the first injection of adrenalin gave each time a slow and gradual rise of pressure, the latent period being respectively 150 and 52 seconds. In the first experiment the blood pressure rise equaled 59 mm. and lasted longer than 15 minutes. In the second experiment (Experiment 6) the first injection into the tracheal sac gave a rise of only 16 mm. of mercury after a latent period of 52 seconds, the rise persisting for more than 15 minutes. A subsequent repetition of the injection gave no blood pressure effect within 6 minutes. The doses of adrenalin injected were respectively 0.3 and 0.25 cc. per kilo.

These experiments show that while absorption of adrenalin does take place from the tracheal mucosa, and therefore probably also from the bronchi and bronchioles, these surfaces play only a subsidiary part as sites of absorption when adrenalin is injected into the trachea.

Section of the vagi in the neck before the intratracheal injection of adrenalin yielded interesting results. These nerves were cut in order to prevent the occurrence of those profound blood pressure drops mentioned previously, on the assumption that they were due to the well known initial effect of adrenalin upon the vagus center. However, these drops still occurred in some of the experiments after section of the vagi, and in addition, pulmonary edema and sudden death were much more frequent than in the series with vagi intact. One intratracheal injection of adrenalin often sufficed to bring on a strong pulmonary edema and even death within a few

minutes.   Without entering here into a discussion of all the phenom-
ena observed, it may be said, in general, that section of the vagi
produced no noteworthy alteration in the absorptive power of the
lung tissue as far as adrenalin is concerned.   The variations observed
in the vagotomized series fell well within the range of those seen in
the normal series, though a percentage reckoning of all intratracheal
injections given for the first time, shows that the vagotomized animal
exhibited a shorter latent period (10 against 13 seconds), a higher
blood pressure rise (56 against 46 mm.), and a longer duration of the
pressure elevation (9.5 against 6 minutes).   Not much stress, how-
ever, should be laid upon averages gained from only twenty-three
experiments, especially in this work where pulmonary edema entered
as a complicating factor in the vagotomy series.

A number of experiments were also carried out with a 0.1 per cent
solution of adrenalin made from the commercial powder, a few drops
of concentrated hydrochloric acid being added to the sterile saline to
bring about solution.   This solution, with or without the addition of
chloretone, did not give as good results when injected intratracheally
as the solution obtained in the open market; the absorption was
slower and the resultant rise of blood pressure less marked.

A few experiments were also carried out with the sodium salt of
fluorescein.   Solutions of this substance, in 1 or 10 per cent strength,
were injected intratracheally, the dose being 0.3 cc. per kilo of body
weight.   Samples of blood were then taken at regular intervals from
the carotid artery, allowed to clot in small test-tubes, and the serum
was examined for fluorescence.   These experiments also indicated a
rapid absorption though not as striking as with adrenalin; after 15 to
30 seconds the blood samples showed the first detectable green fluores-
cence.   This fluorescence rapidly increased at first, then more slowly,
reaching a maximum after a number of minutes.

DISCUSSION AND SUMMARY.

In the preceding pages we have submitted evidence which shows
that a simple intratracheal injection of a solution in a normally breath-
ing rabbit penetrates within a few seconds to the alveoli, chiefly those
of the left lower lobe; that absorption is rapid and well maintained;

and that the procedure may be repeated effectively a number of times even with a substance like adrenalin which decreases absorption. It was also shown that absorption of adrenalin from the lung could be obtained at a time when double the dose given intramuscularly exerted no blood pressure effect whatever, and that absorption could still take place after the development of pulmonary edema, when there was an undoubted dilution of the injected solution with a serum-containing liquid and when a diminution of the absorptive field had occurred.

The solution injected, after reaching the alveoli, is probably largely taken up by the capillaries of the pulmonary veins. This is indicated by the great rapidity with which an intratracheal injection of adrenalin may cause a rise of blood pressure. In numerous instances, for example, the pressure began to rise less than 5 seconds after the completion of an injection, equaling and even surpassing in rapidity of effect an intramuscular injection. Absorption by the lymphatics probably plays a secondary part, an assumption rendered all the more likely if we consider that lymph nodes are interpolated in the lymphatic pulmonary path, where the bed of the lymph stream becomes greatly widened and the current slowed.

Injection into the lungs, however, offers another advantage due to the vascular arrangement of the absorbing field which could be of value therapeutically. Absorption of liquids injected into the lung probably takes place largely through the capillaries of the pulmonary veins; to a slight extent possibly through the capillaries of the bronchial veins which empty partly into the pulmonary veins, partly into the azygos veins; and probably some absorption occurs also through the lymphatics. By far the larger proportion of the absorbed material will thus be rapidly delivered to the left auricle and then to the left ventricle. At each succeeding systole, as long as absorption continues, a fraction of the drug will be driven into the coronary arteries and be able to affect the musculature of the cardiac pump. This fact ought to render the procedure of intratracheal injection a valuable method when it becomes imperative to stimulate a suddenly failing heart as promptly as possible by drugs of the digitalis group.

Intratracheal injection is perhaps better under the conditions mentioned than the intravenous route, for the surface veins cannot

always be entered with promptness and certainty even under fairly normal conditions, and in cases of cardiac weakness the difficulties will be measurably increased, while an intratracheal injection can be carried out with ease. Moreover, it is legitimate to expect that some absorption will take place from the lung alveoli as long as the heart-lung circulation persists, no matter how feebly, and that thus some of the drug will reach the heart to act on this structure itself more promptly perhaps than when the drug is administered successfully through surface veins. As far as the intramuscular route is concerned, we have shown that the intratracheal injection of adrenalin gives prompt though diminished absorption at a time when double the dose intramuscularly exerts no blood pressure effect whatever.

The technical difficulties of giving an intratracheal injection in animals are slight. Tracheotomy as practised by us in the present series of experiments is not necessary, for the injection may be given into the intact trachea without exposure of the trachea. The hypodermic needle is inserted through the skin about 1 cm. below the larynx in a slanting caudad direction; the entrance of the needle into the trachea is readily felt. The injection should not be so rapid that the injected solution fills the entire tracheal lumen, but it should flow down the sides of the trachea. If the lumen is entirely filled, an expiration may drive some of the injected liquid into the larynx causing cough. In our experiments each injection of about 0.5 cc. consumed approximately 5 seconds.

In the human subject no data are available as far as our knowledge goes, but *a priori* it would seem that an intratracheal injection is almost as simple as in the lower animals. The free hypodermic needle could be inserted into the tracheal lumen immediately below the cricoid cartilage. The needle itself should preferably be connected with the syringe by a short length of rubber tubing to minimize the danger of breaking the needle by a sudden move of the patient. The amount of the solution should not be too small, so that at least a fraction of it may reach the alveoli as promptly as possible; 3 to 5 cc. probably would suffice.

Insertion of the needle in the locality mentioned would puncture the isthmus of the thyroid, but this is of no significance, especially when the procedure is employed in cases of cardiac failure where the

gravity of the condition would warrant incurring much heavier risks than a slight bleeding from the thyroidal isthmus.

In conclusion it may be said that the incorporation of drugs by intratracheal injection, while not as generally applicable as other methods, nevertheless has advantages which warrant its use also in human therapeutics.

CEPHALIN.

II. BRAIN CEPHALIN.[1]

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In recent years a number of important contributions to the chemistry of cephalin have appeared, particularly those by Parnas and his coworkers.[2] Through the efforts of these workers the principal components have been identified as cephalinic acid ($C_{19}H_{32}O_2$), stearic acid, aminoethyl alcohol, glycerol, and phosphoric acid. Assuming that the molecule of cephalin is composed of equimolecular proportions of these components, we may expect the following composition of the substance:

$$C_{41}H_{78}NPO_8, \text{ with } C = 66.17; \ H = 10.57; \ N = 1.88; \ P = 4.17$$

There is, however, a considerable discrepancy between these theoretical values and those found by most observers. The discrepancy, of course, could be explained by the fact that the physical properties of cephalin are of a nature which does not permit the preparation of a sample in sufficient purity. This view, however, is not tenable in the light of our experience with lecithin. Lecithin, both in physical and chemical properties, resembles cephalin closely, and yet the empirical analytical values obtained on the substance by various observers always agree with the theory.

On the other hand, if the empirical composition is correct then the present information regarding the nature of the components of cephalin has to be regarded as incomplete.

In view of this the work on cephalin was resumed, principally from the two following standpoints: (1) Is the composition of cephalin

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913–14, xvi, 419.

[2] Parnas, J., *Biochem. Z.*, 1909, xxii, 411. Baumann, A., *ibid.*, 1913, liv, 30. Renall, M. H., *ibid.*, 1913, lv, 296.

Author.	Source.	C	H	N	P	NH₃	Me
Thudichum[3]..........	Brain	60.00	9.38	1.68	4.27		
Zuelzer[4]...............	Ox brain	60.20	9.80	3.80	2.60		
Koch[5]................	Sheep brain	59.50	9.80	1.75	3.85		1.73
Cousin[6].............	Brain	.		1.82	3.89		
Stern and Thierfelder[7].	Egg yolk	59.68	9.74	1.57	3.64		
Falk[8]................	{ Nerves	55.75	9.66	1.94	4.42		
	{ Human brain	57.56	9.21	2.93	3.23		
Neubauer[9]...........	Brain {	61.99	9.85	1.65	3.44		
	{	62.12	9.87	1.69	3.45		
Parnas[10]..............	Brain			1.83	3.86		
Frank[11]..............	Liver	57.10	9.62	1.72	3.91		
Baumann[12]..........	Human brain			1.84		1.63	
Renall[13].............	{ Ox brain			1.69	3.56	1.37	
	{ Sheep brain			1.92	4.27	1.60	
Bürger and Beumer[14]..	Erythrocytes			1.78	4.06		

constant? (2) Do the quantities of the components obtained on hydrolysis agree with those required by the theory, if the prevailing hypothesis of the structure of cephalin is correct?

By varying the nature of the organic solvents and of the temperature at which precipitation or extraction took place we could not change the composition of the resulting phosphatide. Attempts were then made to purify the substance through the lead salt. The analysis of the lead salt gave values for cephalin identical with those of the original substance. When the free cephalin was liberated from the lead salt it again manifested a composition identical with that of the

[3] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884, 57.

[4] Zuelzer, G., Z. f. physiol. Chem., 1899, xxvii, 262.

[5] Koch, W., Z. f. physiol. Chem., 1902, xxxvi, 136.

[6] Cousin, H., Compt. rend. Soc. de biol., 1906, lxi, 23.

[7] Stern, M., and Thierfelder, H., Z. f. physiol. Chem., 1907, liii, 381.

[8] Falk, F., Biochem. Z., 1908, xiii, 163; 1909, xvi, 190.

[9] Fränkel, S., and Neubauer, E., Biochem. Z., 1909, xxi, 321.

[10] Parnas, Biochem. Z., 1909, xxii, 411.

[11] Frank, A., Biochem. Z., 1913, l, 273.

[12] Baumann, Biochem. Z., 1913, liv, 30.

[13] Renall, M. H., Biochem. Z., 1913, lv, 296.

[14] Bürger, M., and Beumer, H., Biochem. Z., 1913, lvi, 450.

original material. Finally, the free phosphatide obtained through the lead salt was reconverted into the lead salt, and this again did not alter the composition of the resulting substance. On the basis of this, one is inclined to conclude that so called cephalin contains some other substance besides the enumerated components. The results of the quantitative hydrolysis of a purified sample showed that the sum of the found components averaged only 90 per cent of the original weight of the substance.

There is nothing to force the conclusion that the substance unaccounted for is an integral part of the molecule. It may be an impurity. We, therefore, concluded to make an attempt to convert cephalin into a saturated phosphatide by reduction with hydrogen after Paal. These experiments were under way before the appearance of Paal's work on the hydrogenation of lecithin.[15] Unfortunately the behavior of the substance towards the reducing agent is very disappointing.

The reduction of cephalin proceeds at a very slow rate, so that for the present we are in possession of only small quantities of material.

The samples thus far analyzed gave analytical values analogous to those of the original material. However, the experience on sphingomyelin has shown that often thirty recrystallizations are required before the substance is obtained in a sufficient degree of purity. Hence we hope to reach a conclusion regarding the true composition of cephalin through further purification of the hydro derivative. Work towards that end is in progress.

EXPERIMENTAL PART.

Preparation of Cephalin.

The cephalin-lecithin fraction of the brain, representing the ether-soluble portion of the alcoholic extract, which still contained some of the cerebroside fraction (cerebrin and sphingomyelin) and some cholesterol, was first purified by precipitating a concentrated ether or petroleum ether solution with dry acetone and then repeatedly with 95 per cent alcohol. After the second or third precipitation from alcohol the precipitate contained little or no ether-insoluble material (cerebroside fraction). The composition of the cephalin did not

[15] Paal, C., and Oehme, H., *Ber. d. chem. Ges.*, 1913, xlvi, 1297.

change after the fifth or sixth precipitation. This material was then either twice suspended in absolute acetone and stirred vigorously for about 2 hours to completely dehydrate it, or better, precipitated two or three times from a concentrated ether solution with dry acetone. Under these conditions the cephalin precipitated as a slightly yellow granular powder, which gradually darkened upon drying, forming a light or dark brown powder. This was easily pulverized. While the moist cephalin is hygroscopic the dried product is stable and easily handled. Prepared in this way cephalin contains a varying amount of ash.

An ash-free preparation may be obtained as follows: The cephalin is emulsified with water by grinding it with a small amount of water in a mortar to a fine paste and then adding water, with stirring, until a thin emulsion results. This is then precipitated by adding 10 per cent hydrochloric acid until no further precipitate forms. The cephalin separates as a light yellow amorphous mass which filters slowly with suction. It is best separated from the water by centrifuging the mixture. The moist product is taken up in dry ether and the solution either dried with sodium sulphate, concentrated, and precipitated, or better, concentrated at once and precipitated with dry acetone.

The same object is more rapidly accomplished by decanting off most of the dilute acid, filtering the cephalin over cheese-cloth, and extracting the cephalin with ether. The difficulty of extracting with ether, in that it forms a gelatinous emulsion,[16] is overcome by the addition of absolute alcohol, care being taken that the amount added does not precipitate the cephalin.

In either case repeated precipitation is necessary to obtain an anhydrous product. This method is accompanied by considerable loss of material, because of the solubility of the cephalin in the acidulated water.

The same object is obtained, probably with less loss of material, by shaking an ether solution of cephalin with 10 per cent hydrochloric acid solution several times, according to Parnas,[17] then with

[16] Thudichum, *loc. cit.*, 58.
[17] Parnas, *loc. cit.*

water, and precipitating as above. The emulsions which may result are best prevented by the addition of absolute alcohol. Such a sample gave the following figures upon analysis:

0.1542 gm. substance gave 0.3420 gm. CO_2 and 0.1312 gm. H_2O.
0.1516 "   "   " . 0.3342 "   "   "   0.1250 "   "
0.5000 "   "   neutralized 7.0 cc. $\frac{N}{10}$ HCl.
0.2000 "   "   in acetic acid, gave 6.4 cc. N gas at 20° and 750 mm.
0.3000 "   "   gave 0.0410 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{78}NPO_8$:	Calculated[18] for $C_{41}H_{78}NPO_{13}$:	Found:
C...................................	66.17	60.00	60.49
			60.13
H...................................	10.57	9.55	9.52
			9.22
N...................................	1.88	1.70	1.96
HN_2N...............................	1.88	1.70	1.79
P...................................	4.17	3.76	3.80

The formula $C_{41}H_{78}NPO_8$ is based on the assumption that the molecule contains 1 molecule each of aminoethyl alcohol, $H_2NCH_2CH_2OH$, glycerophosphoric acid, $C_3H_5(OH)_2OPO(OH)_2$, stearic acid, $C_{18}H_{36}O_2$, and cephalinic acid, $C_{18}H_{32}O_2$. As is seen from the analytical figures, and as has already been mentioned in the introduction, there is a marked discrepancy between these figures and the calculated ones. The formula $C_{41}H_{78}NPO_{13}$ agrees much better with the analytical figures found.

Attempts to Purify Cephalin.

Thinking that perhaps our methods of purification were not sufficiently rigorous, we submitted our material to the following treatments.

A portion of cephalin was dissolved in boiling amyl alcohol (50 gm. require about 250 cc. for solution) and the solution quickly cooled. The cephalin separated as a thick, amorphous mass. This was ground up with dry acetone and repeatedly washed with the same solvent.

A second lot was dissolved in hot ethyl acetate (10 gm. require about 200 cc. for solution). Upon cooling the solution in the ice

[18] Fränkel and Neubauer, *Biochem. Z.*, 1909, xxi, 321.

box the cephalin precipitated out nearly quantitatively. This was washed with dry acetone as above. The composition of the cephalin in either case remained unchanged, so that there was no purification by these methods. This is in agreement with the observations of Baskoff[19] on liver heparphosphatide.

We then examined the method used successfully by MacLean[20] in obtaining cuorin. This consists in extracting the crude material with alcohol at 60–70° for several hours and repeating the process several times. Finding that this operation did not give us any purification, we modified the method as follows. The cephalin was dissolved in a small amount of gasoline, boiling at 50–60°, or in ether, and poured into 98 per cent alcohol which was kept at about 60° during the operation. A part of the cephalin settles out as a thick oil on the bottom and sides of the flask and on the stirrer, while a part remains in the alcohol-gasoline or the alcohol-ether mixture. The precipitate is taken up in ether and precipitated with acetone. The solution is concentrated in vacuum, the residue taken up in ether and precipitated. Since the two lots of material have the same composition no fractionation was effected. The following figures were obtained upon analysis of the fraction which remained in the alcohol-ether mixture:

0.1482 gm. substance gave 0.3260 gm. CO_2 and 0.1274 gm. H_2O.
0.5000 "   "   neutralized 6.33 cc.$\frac{N}{10}$ HCl.
0.3000 "   "   gave 0.0388 gm. $Mg_2P_2O_7$.

	C	H	N	P
Found:	60.00	9.62	1.78	3.60

Properties of Cephalin.

There is little to add to the many properties already given by Thudichum, Falk, Parnas, and Fränkel. It may be well to call attention to certain differences which have been noted in the literature. Thudichum states that cephalin is soluble in anhydrous ether, while Parnas[17] says it is not. We have found that perfectly anhydrous ether does not dissolve cephalin, but remains clear for

[19] Baskoff, A., Z. f. physiol. Chem., 1908, lvii, 395.

[20] MacLean, H., Z. f. physiol. Chem., 1908, lvii, 304; Biochem. Jour., 1909, iv, 168; 1912, vi, 333.

some time. A drop of water added to the ether at once produces the characteristic deep red, fluorescent solution.

The above mentioned method of purification shows that cephalin is markedly soluble in warm 98 per cent alcohol (it does not precipitate out when the ether is removed). Koch and Falk state that it is insoluble in cold or hot alcohol. This may be influenced by the fact that the cephalin is added as an ether solution; the same concentration cannot be reached by boiling cephalin with alcohol. That cephalin is somewhat soluble in cold alcohol is easily seen in the loss on repeated precipitation.

The precipitation of cephalin from an aqueous emulsion or suspension by hydrochloric acid is probably a process of coagulation and does not depend upon the formation of a hydrochloride.[21] Fränkel and Neubauer[18] express the same view and show that the same precipitation is brought about by organic acids and mineral salts.

Cephalin may be obtained in suspension as a nearly colorless substance by precipitation with hydrochloric acid. All attempts to dry it in such a way as to retain this color have failed. As soon as the water is decanted off and acetone is added, the brown color appears. If ether is used, this brown color appears instantly. The color seems to have no influence upon the composition of the molecule.

Lead Compound of Cephalin.

Since the various methods of fractional precipitation and extraction of cephalin failed to give us a product that corresponded to the one theoretically expected, we attempted a purification by chemical means. The most promising method seemed to be through the lead compound. Such a derivative of cephalin (peroxycephalin) has been described by Thudichum.[22] He prepared it by treating an ether solution of cephalin with a warm alcoholic solution of lead acetate and purified it by precipitating from ether with alcohol.

The lead compound of cephalin was prepared as follows: 20 gm. of cephalin were dissolved in 200 cc. of boiling amyl alcohol and this hot solution was treated with a boiling solution of about 25 gm.

[21] Thudichum, *loc. cit.*, 53.
[22] Thudichum, *loc. cit.*, 139.

of lead acetate in amyl alcohol. The deep red solution was then allowed to cool nearly to room .temperature and an excess of methyl alcohol added, with stirring, which precipitated the lead compound as a light yellow amorphous powder. This can be easily dried and is not hygroscopic. The first samples prepared seemed to have a nitrogen to phosphorus ratio of 1: 2, but later preparations, carefully purified, had approximately the same ratio as the original cephalin. The material is purified by thoroughly extracting with boiling methyl alcohol, 80 per cent acetone, and dry acetone. Before purification a part of the lead salt is soluble in ether. The soluble and insoluble parts appeared to have the same composition. After precipitation of the ether-soluble fraction by adding about two volumes of methyl alcohol and extraction of the precipitate with dry acetone, the product is no longer ether-soluble. Thus the apparent solubility in ether was probably due to some impurity. The lead compound is insoluble in cold or warm alcohol, acetone, or ether, more or less soluble in benzene, toluene, and pyridine. Upon cooling the hot amyl alcohol solution deposits the unchanged lead salt as an amorphous mass. The amyl alcohol may be removed by washing with dry acetone. Glacial acetic acid does not completely dissolve the lead salt; it seems likely that a part of the lead is split off during the process of solution, since the residue from the extraction with acetic acid was found to contain lead acetate. A few of the many analyses are given below:

A. 0.1482 gm. substance gave 0.2154 gm. CO_2, 0.0790 gm. H_2O, and 0.0622 gm. ash.
   0.5000 "   "   neutralized 3.16 cc.$\frac{N}{10}$ HCl.
   0.3000 "   "   gave 0.0252 gm. $Mg_2P_2O_7$.
B. 0.1498 "   "   "   0.2188 gm. CO_2, 0.0840 gm. H_2O, and 0.0634 gm. ash.
   0.5000 "   "   neutralized 3.11 cc.$\frac{N}{10}$ HCl.
   0.3000 "   "   gave 0.0294 gm. $Mg_2P_2O_7$.
C. 0.5000 "   "   neutralized 3.21 cc.$\frac{N}{10}$ HCl.
   0.3000 "   "   gave 0.0262 gm. $Mg_2P_2O_7$.
D. 0.5000 "   "   neutralized 3.01 cc.$\frac{N}{10}$ HCl.
   0.3000 "   "   gave 0.0276 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{74}NPO_9Pb_2$:	Calculated for $C_{41}H_{74}NPO_{13}Pb_2$:	A.	Found: B.	C.	D.	Thudichum
C.......	42.65	39.82	39.64	39.84			38.37
H.......	6.46	6.03	5.81	6.27			5.76
N.......	1.21	1.13	0.88	0.87	0.90	0.84	0.97
P.......	2.68	2.50	2.34	2.73	2.43	2.59	2.72
Ash.....			41.97	42.40			

Preparation of Cephalin from the Lead Compound.

In order to determine whether the' preparation and purification of the lead compound had brought about any purification of the cephalin itself, the lead compound was reconverted into the free cephalin.   50 gm. of the material were dissolved in a mixture of 200 cc. of toluene, 100 cc. of benzene, and 50 cc. of pyridine by warming a short time on the water bath.   The lead was removed by means of hydrogen sulphide, the lead sulphide filtered off, and the clear solution concentrated to dryness in vacuum.   The residue was taken up in ether and precipitated repeatedly with dry acetone.   As the analyses show, this material had practically the same composition as the original cephalin used for making the lead salt; this indicates that the lead process is not one of purification.

0.1488 gm. substance gave 0.3276 gm. CO_2 and 0.1278 gm. H_2O.
0.5000 "   "   neutralized 6.84 cc. $\frac{N}{10}$ HCl.
0.3000 "   "   gave 0.0388 gm. $Mg_2P_2O_7$.

	C	H	N	P
Found:	60.05	9.61	1.87	3.60

This process is, at best, very unsatisfactory.   The preparation of the lead salt is not quantitative, and the removal of the lead is accompanied by great loss of material.   The operations must be carried out as rapidly as possible, for if cephalin or its lead compound is allowed to stand for some time in a benzene-pyridine solution, even at 0°, a certain decomposition occurs, in which the nitrogen content is increased.   The nature of this change is being further investigated.

Two samples of cephalin, purified through the lead salt, were again converted into the lead compound as given above.   The same lead compound was obtained, again showing that the cephalin thus obtained was identical with that used for the preparation of the original lead salts.

0.5000 gm. substance neutralized 3.4 cc. $\frac{N}{10}$ HCl.
0.5000 "   "   "   3.46 " " "
0.3000 "   "   gave 0.0242 gm. $Mg_2P_2O_7$.
0.3000 "   "   "   0.0260 "   "

	N		P	
Found:	0.95	0.97	2.24	2.41

Quantitative Hydrolysis.

As the base and the glycerophosphoric acid of cephalin are water-soluble, it is comparatively easy to determine the amount of fatty acid obtained upon hydrolysis. 5 gm. of cephalin were boiled with 300 cc. of 3 per cent sulphuric acid for 24 hours. Upon cooling to 0°, the fatty acids solidified. These were filtered off, washed free of acid by melting over water several times, and then taken up in dry acetone. By evaporating off the acetone several times the product was dehydrated. It was finally dried to constant weight at 100°.

4.75 gm. cephalin gave 2.970 gm. fatty acid.
2.75 "   "   "   3.026 "   "   "
5.0 "   "   "   3.202 "   "   "

		Fatty acid. *per cent*	
Found:	62.6	63.7	64.0

Glycerol Estimations.

Koch believed that cephalin contained one methyl group attached to nitrogen, because when heated with hydriodic acid one molecule of methyl iodide was formed under 240°. Similar results were obtained by Fränkel and Neubauer. Renall also reports the formation of methyl iodide when cephalin was heated to 360°, but states that the amount obtained was not equivalent to. the ratio $N:Me = 1:1$. Recent work makes it appear evident that this formation of methyl-iodide was really due to the presence of glycerol.

Foster[23] has shown that glycerol may be readily determined in lipoids by the use of the original Fanto-Zeisel method. By using a modified Herzig-Meyer apparatus it is possible to determine both the glycerol and the methyl attached to nitrogen.

We have used the Fanto-Zeisel method for the determination of glycerol in cephalin with satisfactory results. The reaction proceeds smoothly, with little or no foaming, and is complete in about 2 hours if the temperature of the metal bath is maintained at 125–128°. Blank determinations on a known solution of glycerol in water gave good checks. Experiments with aminoethyl alcohol showed that

[23] Foster, M. L., *Jour. Biol. Chem.*, 1915, xx, 403.

under the conditions of the reaction no decomposition (with the formation of volatile iodides) took place. 0.3000 gm. of cephalin gave, in three experiments, 0.0748, 0.0760, and 0.0784 gm. of silver iodide respectively. This corresponds to 9.77, 9.93, and 10.2 per cent glycerol. Koch's result, as recalculated by Foster, was 10.8 per cent, while Winterstein and Hiestand found 10.2 per cent. Foster obtained a slightly lower figure, 8.21 per cent. The corresponding figure for lecithin varies from 8.75 to 11.6 per cent glycerol.

Composition of the Cephalin Molecule.

The amount of base (aminoethyl alcohol) in cephalin cannot be estimated directly, because all its derivatives (gold chloride salt, platinic chloride salt, picrate, picrolonate) are very soluble in water or alcohol. However, since all the nitrogen has been shown to be amino nitrogen,[24] we are justified in using the percentage of nitrogen as a basis for the calculation of the amount of base. With 1.9 as the average of the nitrogen content, the amount of aminoethyl alcohol is calculated as 8.26 per cent.

In the same way, with 3.85 as the average phosphorus content, the amount of phosphoric acid is calculated as 12.17 per cent.

In calculating the composition of the molecule the components should add up to 109.7 per cent, since this is the relation between the molecular weight of the formula $C_{41}H_{78}NPO_8$ and the hydrated formula $C_{41}H_{86}NPO_{12}$. The calculated and found values are given below:

	Calculated for $C_{41}H_{78}NPO_8$:	Found:
Base	8.2	8.26
Glycerol	12.4	10.00
Phosphoric acid	13.2	12.17
Fatty acid	75.9	63.40
Totals	109.7	93.83

Thus it is seen, as mentioned in the introduction, that the molecule is not entirely accounted for. As yet we can give no reason for the discrepancy.

[24] Baumann, *Biochem. Z.*, 1913, liv, 30. Renall, *ibid.*, 1913, lv, 296.

Hydrocephalin.

1 gm. of cephalin was dissolved in a mixture of 40 cc. of ordinary ether and 5 cc. of glacial acetic acid and shaken with colloidal palladium in an atmosphere of hydrogen, according to Paal. The hydrogen was absorbed at the rate of about 1 cc. per minute, gradually decreasing as the reaction proceeded. At the end of the reaction 70 cc. (measured at room temperature) had been absorbed, while 1 gm. of cephalin, with the formula $C_{41}H_{78}NPO_{13}$ (mol. wt. 743) should absorb 60 cc. measured under standard conditions. Reduction experiments in pyridine and acetic acid or in amyl alcohol and acetic acid were unsuccessful. The reaction product was concentrated on the water bath until all the ether had been removed, then treated with a large volume of acetone and filtered. The precipitate was taken up in boiling absolute alcohol containing a trace of acetic acid, heated until the palladium was coagulated, and filtered. Upon cooling the reduced cephalin separated out as an amorphous powder. This was purified by washing with ether, dissolving in boiling absolute alcohol, and allowing the product to settle out at 0°. It forms a nearly colorless, non-hygroscopic powder. Because of the small amount at our command we were unable to obtain it crystalline.

Two samples were analyzed. The first was analyzed after the second and third crystallizations, the second after the second crystallization.

A. 0.0998 gm. substance gave 0.2273 gm. CO_2 and 0.0930 gm. H_2O.
   0.1062 "   "   " 0.2420 "   "   " 0.0958 "   "
   0.0300 "   "   " 0.0700 "   "   " AgI (glycerol estimation).
B. 0.1060 "   "   " 0.2412 "   "   " 0.1004 gm. H_2O.

		C	H	Glycerol.
Found:	A.	62.12	10.43	9.15
		62.15	10.10	
	B.	62.06	10.60	

The two samples given above were prepared last year. This fall we again took up the question of obtaining hydrocephalin, but with no better success. The reaction appears to be uncertain; at times the reduction proceeds smoothly, at other times scarcely any hydrogen is absorbed. We have tried to further purify the cephalin but this does not increase the rate of reduction.

The product obtained this year gave the following figures on analysis:

0.1022 gm. substance gave 0.2256 gm. CO_2 and 0.0912 gm. H_2O.
0.300 "   "   " 0.0370 " $Mg_2P_2O_7$.
0.200 "   "   neutralized 3.01 cc. $\frac{N}{10}$ HCl.

	Found:
C.	62.25
H.	10.34
N.	2.22
P.	3.62

GLUCOSAMINOHEPTONIC ACID.

BY P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The work of Levene and La Forge[1] has demonstrated that the substitution of the amino group by a hydroxyl in the series of amino sugars may be associated with a change of configuration of the α-carbon atom. This property raises great difficulty in reaching, by simple methods, a conclusion regarding the complete configuration of any amino sugar. The configuration of glucosamine, the most accessible and the best known substance of this group, still remains unknown. Because of the difficulty of arriving at the goal by direct methods, the problem is being approached in this laboratory by several indirect ways, all of which will be discussed later. One of these, however, will be outlined here. In the light of the experience of Fischer[2] and his coworkers it seems possible that a substitution in the β position from the carbonyl group may take place without a Walden rearrangement. If this were to hold true for the 2-aminoheptonic acids, then the solution of the question of the configuration of these substances should be easily accessible. On the other hand, the knowledge of the configuration of the carbon atom carrying the amino group in the heptonic acids will contain in itself the information regarding the configuration of the same group in the parent hexosamines. Hence the first problem demanding solution is the question of the occurrence or non-occurrence of the Walden rearrangement during deamination of the 2-aminoheptonic acids. The derivative of glucosamine was the subject of the present investigation. In

[1] Levene, P. A., and La Forge, F. B., *Jour. Biol. Chem.*, 1915, xx, 433; xxi, 345, 351; xxii, 331.

[2] Fischer, E., and Scheibler, H., *Ber. d. Chem. Ges.*, 1909, xlii, 1219. Fischer, E., Scheibler, H., and Groh, R., *ibid.*, 1910, xliii, 2020.

order to make this investigation possible it is necessary to be in possession of a crystalline 2-aminoheptonic acid derived from glucosamine.

An attempt to prepare this heptonic acid was made by Neuberg,[3] who claimed to have obtained an amorphous copper salt, which gave analytical values agreeing with those required by the heptonic acid. Such material could be of little value in our work, especially since the amorphous salts prepared by us gave values only approximating those required by the theory. Hence we renewed the efforts towards obtaining a crystalline glucosaminoheptonic acid. The present communication contains a report of the mode of preparation and of the properties of the acid which has the structure of one of the following two possible epimers.

$$CH_2(OH)\frac{H\quad H\quad OH\quad H\quad H}{OH\quad OH\quad H\quad NH_2\quad OH}COOH \text{ or}$$
$$(?)$$

$$CH_2(OH)\frac{H\quad H\quad OH\quad H\quad OH}{OH\quad OH\quad H\quad NH_2\quad H}COOH$$
$$(?)$$

EXPERIMENTAL PART.

Portions of 25.0 gm. of glucosamine hydrochloride were taken up in 25 cc. of water, 10 cc. of an 80 per cent aqueous solution of hydrocyanic acid added, and this was followed by the addition of 12.5 cc. of ammonia water. The chloride generally dissolved soon after the addition of ammonia. The solution turned dark brown within 2 hours, and on further standing became quite viscous. It was allowed to stand at room temperature for 3 to 4 days. The solution was then taken up in about 400 cc. of water, boiled with a slight excess of lead carbonate, filtered, and precipitated with basic lead acetate and ammonia. The precipitate was decomposed with hydrogen sulphide, filtered, and concentrated to about 300 cc. To the solution about 40.0 gm. of barium hydroxide were added and the mixture was heated over a flame until the greatest part of ammonia was removed. On standing, a brown flocculent precipitate formed

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[3] Neuberg, C., *Ber. d. Chem. Ges.*, 1902, xxxv, 4009. Neuberg, C., and Wolff, H., *ibid.*, 1903, xxxvi, 618.

and was removed by filtration. The filtrate was then concentrated under diminished pressure to dryness to remove all the ammonia. The residue was taken up in water, and the solution acidulated with sulphuric acid to remove all barium. Omitting filtration, excess of lead carbonate was added and the mixture boiled until, upon a further addition of the reagent, evolution of carbon dioxide was no longer manifested. The reaction product was then allowed to stand at 0°C. over night and filtered. The lead was then removed by means of hydrogen sulphide, the remaining hydrochloric acid by means of silver carbonate, and the excess of silver again removed by hydrogen sulphide. The filtrate was boiled with animal charcoal until it became perfectly colorless. The colorless solution was concentrated to about 75.0 cc. and to this methyl alcohol was added in small portions. The addition of alcohol caused the appearance of a white flocculent precipitate at the place of contact. By manipulation this was slowly brought again in solution, and the addition of alcohol continued until the solution turned slightly opalescent. It was then allowed to stand at least 24 hours at 0°C. A crystalline precipitate appeared, consisting of rosettes composed of needles. The yield was about 30 per cent of the glucosamine hydrochloride used. On recrystallization the substance crystallized in individual needles. The acid has a melting point of 169°C. (corrected). For analysis it was dried in a vacuum desiccator over sulphuric acid at 100°.

0.1004 gm. of substance gave 0.1366 gm. CO_2 and 0.0618 gm. H_2O.
0.0970 " " " required for neutralization 4.38 cc. $\frac{N}{10}$ acid (Kjeldahl).

	Calculated for $C_7H_{13}NO_7$:	Found:
C..	37.33	37.10
H..	6.66	6.88
N..	6.22	6.25

In 2.5 per cent solution of hydrochloric acid the substance had the following rotation:

Initial.

$$[\alpha]_D^{25} = \frac{-0.02° \times 2.168}{0.1 \times 0.150} = -0.289°$$

Equilibrium.
(After 24 hours.)

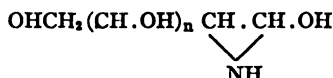$$[\alpha]_D^{25} = \frac{-0.52° \times 2.168}{0.1 \times 0.150} = -7.52°$$

AMMONIA DERIVATIVES OF THE SUGARS.

BY P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The structure of the so called glucosimines has been the subject of considerable discussion. Lobry de Bruyn,[1] who first discovered them, assigned to them the following structure:

$$OHCH_2(CH.OH)_n \underset{NH}{CH.CH.OH}$$

Wohl[2] modified this first conception, accepting the ring between the end and the γ-carbon atoms:

$$OHCH_2CH(OH)CH.CH(OH)CH(OH)CH.OH$$
$$|\underline{\qquad NH \qquad}|$$
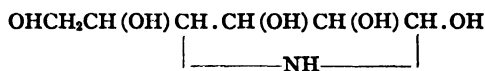
Finally, Irvine, Thomson, and Garret[3] have assumed the glucosidic structure:

$$OHCH_2CH(OH)CH.CH(OH)CH(OH)CH.NH_2$$
$$|\underline{\qquad O \qquad}|$$
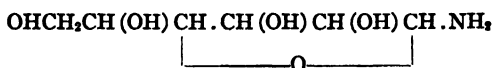
The evidence advanced by Irvine and his coworkers is of indirect nature, and more direct evidence supporting their hypothesis seemed desirable. Besides, the knowledge of the structure of these substances has assumed new importance because of its bearing on the mechanism of the reaction between hydrocyanic acid and the sugar imines.

[1] Lobry de Bruyn, C. A., and Franchimont, A. P. N., *Rec. d. trav. chim. d. Pays-Bas*, 1893, xii, 286. Lobry de Bruyn, *Ber. d. chem. Ges.*, 1895, xxviii, 3082.

[2] Wohl, quoted by von Lippmann, E. O., Die Chemie der Zuckerarten, Braunschweig, 1904, i, 504.

[3] Irvine, J. C., Thomson, R. F., and Garret, C. S., *Jour. Chem. Soc.*, 1913, ciii, 238.

In order to prove the glucosidic structure it is necessary to furnish conclusive evidence as to one of two requisites, or to both: first, as to the existence of the γ-oxidic ring in sugars (this structure carries the property of mutarotation); second, as to the presence of an unsubstituted NH_2 group. These two points have been taken into consideration by previous workers, but they received no conclusive answer.

The presence of a primary NH_2 group was tested in this work by comparing the nitrogen values obtained by the Kjeldahl process and by the method of Van Slyke with nitrous acid. It was found that in 4 minutes all the nitrogen of the imine was transformed into nitrogen gas. In the same interval ammonia does not give off more than 20 per cent of its nitrogen as nitrogen gas.[4]

Furthermore, on material which was only slightly hydrolyzed, the values obtained by the Kjeldahl process were higher than those obtained in 4 minutes by the nitrous acid method. In connection with this it may be mentioned that the rate of hydrolysis varies with individual sugars. The derivatives of four sugars were analyzed: glucose, galactose, xylose, and lyxose. Lyxosimine was found the most, and glucosimine the least stable.

Regarding the change in rotation the following was noted. Freshly prepared samples of xylosimine and of lyxosimine, kept in aqueous solution at 0° for 24 hours, did not manifest any change in rotation. In 63 hours there was observed a slight change in rotation and with it there was also noted a change in nitrogen distribution, the nitrogen gas evolved by nitrous acid showing a lower value than the total nitrogen.

On the other hand, if the sample of the dry imine was kept for 7 days over soda lime under diminished pressure and then analyzed, it was found to manifest some mutarotation in 24 hours, even when the nitrogen distribution remained constant. In this sample there was always present a slight excess of the total nitrogen over the amino nitrogen. Hence the mutarotation was undoubtedly due to the presence of little of the free sugar.

Thus, the presence of the primary amino group in the molecule of the sugar imines is demonstrated; on the basis of this one is justi-

[4] Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 281.

fied in assigning to the sugar imines a glucosidic structure. The name amino-glucosides would perhaps be more correct.

Table of Results.

	Total N calculated for $C_5H_{11}NO_4 = 9.40$ per cent. Found:		NH$_2$ N calculated for $C_5H_9O_4.NH_2 = 9.40$ per cent. Found:		
	Immedi- ately.	After 63 hrs.	Immedi ately.	After 24 hrs.	After 63 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Xylosimine.					
I......................	9.73	9.64	9.39		9.07
II......................	9.06		8.78	8.78	
Lyxosimine.					
I......................	9.64	9.58	9.16		8.38
II......................	8.92		7.83	7.80	

EXPERIMENTAL PART.

I. Xylosimine[5] was prepared according to the method of Lobry de Bruyn and Van Leent.[6] The substance was dried for 24 hours over soda lime under diminished pressure.

0.1000 gm. substance required for neutralization 6.95 cc. $\frac{N}{10}$ acid (Kjeldahl).
0.0500 "   "   gave in 4 minutes 8.2 cc. N at 20°, 764 mm. (Van Slyke).
The same solution was analyzed after 63 hours' standing at 0°C.
0.1000 gm. substance required for neutralization 6.89 cc. $\frac{N}{10}$ acid.
0.0500 "   "   gave in 4 minutes 7.9 cc. N gas at 20°, 765 mm.
The rotation of the substance in cold water of about 5°C. (dissolved in water at 0°) was the following:

Immediately.   After 63 hours.

$$[\alpha]_D^{25} = \frac{-0.38° \times 4\ 1850}{0.5\ \times 0.2000} = -15.9° \qquad [\alpha]_D^{25} = \frac{-0.38° \times 4.1850}{0.5\ \times 0.2000} = -15.9°$$

II. Xylosimine was prepared in the same manner and kept for 7 days in a desiccator.

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[5] We take this occasion to express our indebtedness to the Lookout Refining Company, Chattanooga, Tenn., who furnished us free of charge the cottonseed hulls which served for the preparation of the xylose used in this work as well as in the work on "Xylohexosaminic Acid."

[6] Lobry de Bruyn, C. A., and Van Leent, F. H., *Rec. d. trav. chim. d. Pays-Bas*, 1895, xiv, 134.

0.1000 gm. substance required for neutralization 6.47 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 7.7 cc. N gas at 20°, 762 mm.

After 24 hours the same solution gave:

0.0500 gm. substance gave in 4 minutes 7.6 cc. N gas at 19°, 768 mm.

Rotation under the same conditions as above:

Immediately.	After 24 hours.

$$[\alpha]_D^5 = \frac{-0.42° \times 4.1630}{0.5 \times 0.2000} = -17.48° \qquad [\alpha]_D^5 = \frac{-0.38° \times 4.1630}{0.5 \times 0.2000} = -15.71°$$

III. Lyxosimine was prepared in the manner described by Levene and La Forge.[7] It was kept in the desiccator over night.

0.1000 gm. substance required for neutralization 6.89 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 8.0 cc. N gas at 20°, 764 mm.

The same solution after 63 hours gave:

0.1000 gm. substance required for neutralization 6.84 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 7.3 cc. N gas at 20°, 765 mm..

The rotation was:

Immediately.	After 63 hours.

$$[\alpha]_D^5 = \frac{-1.30° \times 4.1950}{0.5 \times 0.2000} = -54.53° \qquad [\alpha]_D^5 = \frac{-1.06° \times 4.1950}{0.5 \times 0.2000} = -44.46°$$

IV. Lyxosimine was prepared as the sample above, but kept in the desiccator 7 days.

0.1000 gm. substance required for neutralization 6.37 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 6.75 cc. N gas at 19°, 768 mm.

The rotation was:

Immediately.	After 24 hours.

$$[\alpha]_D^5 = \frac{-0.72° \times 4.1952}{0.5 \times 0.2000} = -30.25° \qquad [\alpha]_D^5 = \frac{-0.55° \times 4.1952}{0.5 \times 0.2000} = -23.10°$$

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[7] Levene, P. A., and La Forge, F. B., *Jour. Biol. Chem.*, 1915, xxii, 331.

SPHINGOSINE.

IV. SOME DERIVATIVES OF SPHINGOSINE AND DIHYDROSPHINGOSINE.

BY P. A. LEVENE AND C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Sphingosine has generally been identified by the analysis of the sulphate. Some workers have based their conclusions on the presence of sphingosine when the sample possessed the nitrogen content of sphingosine sulphate or, besides the nitrogen content, showed the required melting point. In the course of the work carried out in this laboratory during the last few years the conviction was gained that the composition of the sulphate is not sufficiently constant to justify conclusions on the basis of analytical data for one or two elements. Thus the differences in the nitrogen and sulphur content of the homologues of sphingosine are within the limits of analytical error. Also the melting point does not offer a sufficient guarantee of its purity. Because of this, attempts were made to prepare other salts of sphingosine and dihydrosphingosine. Picric and picrolonic acids were found to give with the two bases salts of constant composition. However, the solubility of these salts in organic solvents is too great to make them very useful for analytical purposes.

This communication contains also a report of further work on the structure of sphingosine. Levene and West[1] have shown that the probable structure of sphingosine is

$$C_{12}H_{25}CH = CH . CHOH . CHOH . CH_2NH_2$$

The isolation of normal pentadecylic acid from the oxidation products of dihydrosphingosine established the nature of the first 15 carbon atoms. There thus remains as unknown the character

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913–14, xvi, 549; 1914, xviii, 481.

of the two end carbon atoms and the relative positions of the hydroxyl and amino groups. Levene and Jacobs[2] first attempted to answer these questions by trying to reduce dihydrosphingosine to the corresponding amine. Using hydriodic acid in sealed tubes at 125°, they obtained an unsaturated compound, sphingamine, $C_{17}H_{35}N$. The same substance was also obtained when the dichloro derivative was reduced with sodium and alcohol. Because of this it was attempted to modify the conditions of reduction of the base by means of hydriodic acid. An interesting observation was made when the process was carried out in glacial acetic acid solution; under these conditions only one hydroxyl group was reduced, leaving a mono-hydroxymonoamino alcohol. This substance undoubtedly could serve as a starting point for further work on the structure of sphingosine. However, a chance discovery made the material more accessible in another way.

In the course of the present work also the trihydroxy alcohol—dihydrosphingosol—was obtained.

EXPERIMENTAL PART.

Sphingosine Picrolonate.

The sphingosine from 4.5 gm. of sphingosine sulphate, dissolved in a little alcohol, was added to a solution of 3 gm. of picrolonic acid in alcohol. The slightly yellow solution immediately turned a deep red. Boiling water was then added until the solution was turbid; upon cooling the picrolonate separated as a deep red oil, which gradually changed to yellow crystals. It was purified by precipitating several times from an alcoholic solution with boiling water and finally by extracting with ether at room temperature. Sphingosine picrolonate is easily soluble in ethyl alcohol, somewhat soluble in methyl alcohol and acetone, and very slightly soluble in ether. It softens at 81° and melts at 87–89°.

0.1110 gm. substance gave 0.2397 gm. CO_2 and 0.0792 gm. H_2O.

	Calculated for $C_{17}H_{42}N_5O_7$:	Found:
C	58.97	58.90
H	7.88	7.98

[2] Levene, P. A., and Jacobs, W. A., *Jour. Biol. Chem.*, 1912, xi, 547.

Abderhalden and Weil[3] report that they were able to estimate picrolonates by the ordinary Kjeldahl method, only two of the nitrogens of picrolonic acid being converted into ammonia. We tried this method on the above picrolonate but found that the value for N was always high. The following results were obtained:

0.2434 gm. substance required 17.8 cc. $\frac{N}{10}$ HCl.
0.2816 "   "   "   20.4 " " "
0.2021 "   "   "   14.7 " " "

	Calculated for 3N:		Found:	
N	7.65	10.24	10.15	10.19

The determination of nitrogen in *dl*-leucine picrolonate[4] was then tried, for which Abderhalden and Weil give: calculated, 10.65; found, 10.90.

0.1924 gm. substance required 17.6 cc. $\frac{N}{10}$ HCl.
0.1777 "   "   "   15.7 " " "

	Calculated for 3N:		Found:	
N	10.65	12.81	12.38

Blank experiments on picrolonic acid gave values between those calculated for 2N and 3N.

Dibromosphingosine Sulphate.

Sphingosine sulphate, dissolved in chloroform, was treated with a dilute solution of bromine in chloroform at room temperature until the bromine color was permanent. The mixture was allowed to stand several hours, the chloroform removed on the steam bath, and the product washed with water and dilute sodium bisulphite solution. After drying it was recrystallized from acetic acid. Dibromosphingosine sulphate forms a light gray crystalline powder, insoluble in alcohol and ether, soluble in acetic acid and chloroform.

0.1200 gm. substance gave 0.1811 gm. CO_2 and 0.0793 gm. H_2O.

	Calculated for $(C_{17}H_{34}NO_2Br_2)_2H_2SO_4$:	Found:
C	41.30	41.10
H	7.34	7.40

[3] Abderhalden, E., and Weil, A., *Ztschr. f. physiol. Chem.*, 1912, lxxviii, 150.
[4] Levene, P. A., and Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 127.

Dihydrosphingosine Sulphate.

This was prepared by the method already described by Levene and Jacobs. It was found in many experiments, especially if the ether contained alcohol, that the bulk of the dihydro base remained in the acetic acid-water solution with the palladium. It was isolated as follows: The ether layer was removed from the acetic acid and water. The ether was concentrated and the residue added to the dilute acid solution; this was then warmed and filtered to remove the palladium, and the filtrate treated with 10 per cent sulphuric acid as long as the sulphate separated. This was thoroughly cooled, filtered, and recrystallized from dilute alcohol (80 per cent). Dihydrosphingosine sulphate differs from that of sphingosine in that it is much less soluble in absolute alcohol and in chloroform.

Dihydrosphingosine Picrolonate.

This was prepared in the same way as the sphingosine picrolonate. It crystallizes in long, slender, yellow needles, which begin to soften at 110° and melt at 120–121°.

0.1317 gm. substance gave 0.2832 gm. CO_2 and 0.0966 gm. H_2O.

	Calculated for $C_{27}H_{44}N_4O_7$:	Found:
C	58.76	58.79
H	8.22	8.23

Dihydrosphingosine Picrate.

2 gm. of the dihydro base in 25 cc. of hot alcohol were treated with 25 cc. of a saturated alcoholic solution of picric acid, and hot water was added until the solution was turbid. The yellow precipitate was purified by recrystallization from dilute alcohol and finally by extraction with ether. The picrate forms pure yellow crystals, which melt at 88–89°.

0.1096 gm. substance gave 0.2150 gm. CO_2 and 0.0762 gm. H_2O.

	Calculated for $C_{23}H_{40}N_4O_9$:	Found:
C	53.44	53.50
H	7.80	7.78

Dihydrosphingosol (*Trihydroxyheptyldecane*) $C_{17}H_{33}(OH)_3$.

Dihydrosphingosine sulphate was dissolved in boiling acetic acid, cooled to room temperature, and gradually treated with slightly more than the theoretical amount of solid sodium nitrite. A concentrated aqueous solution of nitrite may also be used. After standing several hours the reaction product was concentrated to a small volume, poured into a large quantity of ice water, the precipitate filtered off and recrystallized several times from a small quantity of acetone, finally with the use of animal charcoal. *Dihydrosphingosol* is a colorless, crystalline solid, melting at 54–55° and easily soluble in all organic solvents.

	Calculated for $C_{17}H_{36}O_3$:	Found:
C..	70.01	70.76
H..	12.49	12.58

Hydroxyheptadecylamine, $C_{17}H_{34}(OH)NH_2$.

Hydroxyheptadecylamine was obtained as the reaction product of hydriodic acid upon an acetic acid solution of dihydrosphingosine sulphate either at 100° or at the boiling point of the mixture. The only influence the time of heating had was to decrease the yield, the maximum yield (which was always small) being obtained by 5 to 7 hours' heating. One experiment in which the heating was carried out for 20 hours gave the hydroxy-amine as the only basic reaction product. The other product of this reaction appears to be a hydrocarbon or alcohol, produced by the hydrolysis of the amino group.

10 gm. of dihydrosphingosine sulphate were dissolved in a mixture of 50 cc. of hydriodic acid and 200 cc. of glacial acetic acid, about 1 gm. of phosphonium iodide was added, and the solution heated in a boiling water bath for 5 to 7 hours. The solution was then concentrated in vacuum to a thick syrup, the residue washed with water and taken up in absolute alcohol. Alcoholic hydrochloric acid was added to the solution and the mixture gradually treated with metallic zinc until colorless. This colorless solution was concentrated in vacuum to a small volume and the salts, a mixture of the chloride and iodide, were precipitated with cold water. The free base was obtained by neutralizing the alcoholic solution of the salts with sodium methyl-

ate, evaporating to dryness, and extracting with ether. This was changed into the sulphate by warming with dilute sulphuric acid, the crude product recrystallized from acetone, the non-basic part removed by washing with ether, and the sulphate finally recrystallized from absolute alcohol. It separates as long, slender, colorless needles, forming into rosettes, which melt at 206–208°. Many samples were analyzed, of which two are reported here:

0.1082 gm. substance gave 0.2528 gm. CO_2 and 0.1158 gm. H_2O.
0.1226 "   "   " 0.2893 "   "   " 0.1306 "   "

	Calculated for $(C_{17}H_{37}O)_2H_2SO_4$:	Found:	
C................................	63.69	63.43	63.45
H................................	11.96	11.98	11.93

1.5 gm. of the sulphate were transformed into the free base. For this purpose it was dissolved in methyl alcohol. To the alcoholic solution was added a solution of barium hydroxide in methyl alcohol until it reacted alkaline to phenolphthalein. Acetone was then added to remove excess of barium, the filtrate concentrated in vacuum and recrystallized out of alcohol. The free base crystallized out in beautiful scales of the appearance of hydroxyheptadecylamine.[6] It melts at 85.5°.

The analysis of the substance was as follows:

0.1018 gm. substance gave 0.2756 gm. CO_2 and 0.1220 gm. H_2O.

	Calculated for $C_{17}H_{34}(OH)NH_2$:	Found:
C....................................	75.3	74.12
H....................................	13.68	13.47

The analysis shows the presence of a minimal quantity of dihydrosphingosine in the sample of the reduced base. This undoubtedly could be removed by repeated recrystallization.

[6] Levene, P. A., *Jour. Biol. Chem.*, 1915, xxiv, 79.

SPHINGOMYELIN. III.

By P. A. LEVENE.[1]

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Sphingomyelin was discovered by Thudichum.[2] This investigator established the principal distinction of this phosphatide from those of the lecithin group; namely, the absence of glycerol in its molecule. All other statements of the discoverer regarding sphingomyelin needed revision, since they were based on analysis of material that was not a pure phosphatide or pure phosphatides, but a mixture of these with cerebrosides. Rosenheim and Tebb[3] later improved the method of preparation of sphingomyelin, and had in their possession a much purer material. In a general way they accepted Thudichum's conclusions regarding the structure of the substance. Other investigators who, in course of their work on phosphatides, encountered the substance always dealt with grossly contaminated material.

In a previous communication[1] the writer reported a mode of procedure for preparing sphingomyelin free from cerebrosides, and also the conditions for the hydrolysis of the substance. The following substances were then obtained: (1) phosphoric acid; (2) two fatty acids, cerebronic and lignoceric; (3) three basic substances, choline, sphingosine, and a base of the composition $C_{17}H_{35}NO$. Sphingosine had not been isolated in pure form, and the composition of the third base was rather suggested than definitely demonstrated. Since that publication the work has been continued and extended to the sphingomyelin obtained from other organs; namely, kidney, liver, and the yolk of hen's egg. The method of preparation of the substance was

[1] Levene, P. A., *Jour. Biol. Chem.*, 1913, xv, 153; 1914, xviii, 453.

[2] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884, 105.

[3] Rosenheim, O., and Tebb, M. C., *Quart. Jour. Physiol.*, 1908, i, 297; *Jour. Physiol.*, 1910, xli, p. i.

slightly modified with the result that the sphingomyelin employed in the present work was entirely free from contaminating cerebrin.

The elementary composition and the specific optical rotation of the new material were quite constant.   Sphingomyelin is micro-crystalline in nature.

Employing the old method of hydrolysis the products obtained from the new material differed from those recorded in the earlier work only in the point of the acids.   Cerebronic and lignoceric were the two previously recorded.   Cerebronic acid could not be detected in the course of the present work.   In the older material it un-doubtedly originated in the adhering cerebrin.   Lignoceric acid was identified beyond doubt, and with it was demonstrated the presence of another acid, of lower molecular weight.   From the present data it seems to be a hydroxy acid.   The mixed acids obtained on hydro-lysis of sphingomyelin contain at least 50 per cent of the new acid. However, it was not prepared in a state of satisfactory purity through any of the conventional methods of fractionation, either through fractional crystallization from organic solvents, through the sodium, lithium, or magnesium salts, or through fractional distillation of the esters.   It is hoped, however, that with a larger quantity of material on hand the difficulty will be overcome.

For the bases the old method of hydrolysis gave results which in every way substantiated the older conclusions.   Again choline, sphingosine, and a base $C_{17}H_{35}NO$ were identified.   The assumed composition of $C_{17}H_{35}NO$ was demonstrated to be the correct one; and after reduction with hydrogen and palladium it was shown to possess the structure of hydroxyheptadecylamine $C_{17}H_{34}(OH).NH_2$. The latter base gave up, in 10 minutes, all its nitrogen in the form of nitrogen gas on treatment with nitrous acid in the Van Slyke apparatus, and, further, it formed a diacetyl derivative.   The reduced base will be referred to as sphingine.   In the free state it crystallizes in beautiful plates that have the appearance of cholesterol.   How-ever, in the course of the work sufficient evidence accumulated for the belief that the base was a secondary product formed from sphin-gosine.   In the process of hydrolysis of sphingomyelin with alcohol and water sphingosine loses a molecule of water, giving rise to a base of the nature of anhydrosphingosine,

$$C_{12}H_{25}CH=CHCH=C(OH)CH_2NH_2.$$

The position of the additional double bond in this graphic formula is arbitrary. Owing to the great solubility of the free base or of its sulphate in organic solvents it generally escaped detection, but it always contaminated the sphingosine prepared from sphingomyelin. It was perhaps due to this impurity that Thudichum never succeeded in isolating pure sphingosine from sphingomyelin. He referred to the base isolated by him as an impure sphingosine or a homologue of sphingosine.

In our own work the base escaped detection for a long time. Only after the mixed bases were reduced by hydrogen in the presence of colloidal palladium could their separation be accomplished; namely, through the fractional crystallization of their sulphates from a solution of equal parts of chloroform and amyl alcohol.

The reasons for the belief in the secondary origin of anhydrosphingosine are the following: when the cleavage of sphingomyelin was accomplished through hydrolysis in sealed tubes with 3 per cent sulphuric acid solution, anhydrosphingosine could not be detected, and sphingosine was easily isolated in pure condition. On the other hand, when a sample of pure cerebrin—corresponding to Thierfelder's cerebron—was hydrolyzed by the combined method and the bases were subsequently reduced, in place of dihydrosphingosine, pure sphingine was obtained. There should be recorded here another difficulty in identifying sphingosine. The sulphate which generally serves for identification of the base on recrystallization easily changes its composition, a part of the neutral salts changing into either the basic or the acid salt. The same applies to sphingine. It was therefore found necessary to purify sphingosine and more so dihydrosphingosine and sphingine by converting them into the free bases; these may then be reconverted into the sulphate if desired.

Thus, for the present, sphingomyelin was found to yield on hydrolysis phosphoric acid, two fatty acids, and two bases, choline and sphingosine.

Thudichum and Rosenheim and Tebb accepted in the molecule of sphingomyelin besides the bases and the acids also an alcohol. This view does not agree with the analytical finding of the present work; namely, the cleavage of our material yielded 34 per cent of its weight in the form of sphingosine and 43 per cent in the form

of the organic acids.   These values leave no room in the molecule
of the phosphatide for the alcohol sphingol, and, on the other hand,
they agree with the assumption that a monophosphatide of the
sphingomyelin type is composed of single equivalent proportions of
the components isolated by us.

Hence, for the present one is justified in assuming for a phosphatide
of this type the structural expression,

$$O = P\begin{cases} O - C_{17}H_{32}(OH)\,NH\,.\,COC_{23}H_{47}\text{ (or another acid radicle)} \\ OH \\ O\,.\,C_5H_{13}N(OH) \end{cases}$$
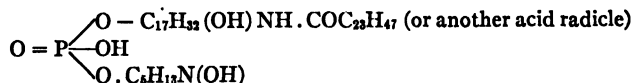
This assumption is further justified by a chance discovery of an
intermediary product.   This was obtained on hydrolysis of a sample
of kidney sphingomyelin.   It possessed the following properties:
it contained nitrogen, gave no nitrogen gas on treatment with nitrous
acid, did not form salts with mineral acids, and had the elementary
composition of lignoceryl sphingine, $C_{17}H_{34}(OH)NH.CO.C_{23}H_{47}$.
Lack of material prevented hydrolysis of the substance.

Whether sphingomyelin is a mixture of two phosphatides of the
described type, or a diphosphatide, can for the present not be stated
with certainty.   This question as well as the question of the structure
of the second fatty acid is under investigation.

Regarding the relation of the sphingomyelins derived from different
organs, one is inclined to believe in their identity.   If ultimately it
will be demonstrated that the sphingomyelin is a mixture of two mono-
phosphatides, then it may appear that their proportion varies some-
what in the material obtained from different organs.   The following
table of the elementary analysis and of the physical properties of
samples derived from different sources brings out best the justification
for the expressed belief.

Source.	C	H	N	P	$[\alpha]_D$	Sphin-gosine.	Acids.
						per cent	*per cent*
Brain........................	66.59	11.26	3.78	3.99	+8.20	34.10	43.00
					+7.53		
Kidney........................	64.80	11.41	3.50	3.82	+8.73	32.10	49.00
Liver........................	64.47	11.57	3.41	3.81	+7.61	32.14	41.70
Egg yolk........................	65.56	11.68	3.84	4.22	+7.54	33.70	43.40

I. Brain Sphingomyelin.[4]

The crude sphingomyelin was prepared according to the directions given in the previous communication. This substance was dissolved in five parts of ligroin and one part of alcohol. Alcohol was then added as long as a precipitate formed. The filtrate was allowed to stand over night at 0°C. and again filtered. The final filtrate was concentrated under diminished pressure and poured into acetone.

The material obtained in this manner was further purified by recrystallization out of a solution in equal parts of pyridine and chloroform. This was carried out in several stages. Five recrystallizations were made at room temperature; these were followed by recrystallizations at 30°, and finally at 37°. The recrystallizations were continued until the substance gave a negative orcin test in the presence of a trace of copper acetate.

Elementary Composition of Sphingomyelin.

I. 0.2835 gm. substance, dried in a vacuum desiccator at the temperature of boiling water, gave 0.0406 gm. $Mg_2P_2O_7$.

0.1890 gm. material required for neutralization 5.1 cc. $\frac{N}{10}$ H_2SO_4.

0.1000 gm. substance gave 0.2430 gm. CO_2, 0.1006 gm. H_2O, and 0.0104 gm. ash.

The optical rotation of the substance dissolved in equal parts of chloroform and methyl alcohol was as follows:

$$[\alpha]_D^{25} = \frac{+0.34° \times 3.6220}{0.5 \quad \times 0.3000} = +8.20°$$

II. 0.3000 gm. substance, dried as Sample I, gave 0.0428 gm. $Mg_2P_2O_7$.

0.2000 gm. substance required 5.2 cc. $\frac{N}{10}$ H_2SO_4.

0.0996 gm. substance gave 0.2438 gm. CO_2 and 0.1012 gm. H_2O.

The optical rotation in chloroform-methyl alcohol solution was:

$$[\alpha]_D^{25} = \frac{+0.34° \times 3.6198}{0.5 \quad \times 0.3000} = +8.20°$$

III. 0.4000 gm. substance, dried as Sample I, gave 0.0564 gm. $Mg_2P_2O_7$.

0.2000 gm. substance required for neutralization 5.1 cc. $\frac{N}{10}$ H_2SO_4.

0.0990 gm. substance gave 0.2402 gm. CO_2 and 0.1044 gm. H_2O.

The optical rotation in chloroform-methyl alcohol solution was:

$$[\alpha]_D^{25} = \frac{+0.31° \times 3.6460}{0.5 \quad \times 0.3000} = +7.53°$$

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[4] The mode of procedure described for the brain tissue was followed in the work on the other organs.

IV. 0.3000 gm. substance, dried as Sample I, gave 0.0434, gm. $Mg_2P_2O_7$.
0.2000 gm. substance required for neutralization 5.2 cc. $\frac{N}{10}$ H_2SO_4.
0.0990 gm. substance gave 0.2410 gm. CO_2 and 0.0998 gm. H_2O.
The optical rotation in the same solution as the other samples was:

$$[\alpha]_D^{25} = \frac{+0.31° \times 3.6404}{0.5 \quad \times 0.3000} = +7.52°$$

Table of Analysis.

	C	H	N	P
I............................	66.27	11.26	3.78	3.99
II............................	66.81	11.30	3.64	3.97
III............................	66.65	11.59	3.57	3.92
IV............................	66.45	11.21	3.64	4.02
Average.....................	66.59	11.34	3.66	3.98

The variations in the elementary composition of the various samples are slight, especially when the size of the molecule and the physical properties of the substance are taken into consideration.

Methods of Hydrolysis.

Two methods of hydrolysis were employed in the course of the work.

One was described in the earlier paper and was carried out in two stages. In the first the substance was heated with barium hydroxide, in the second with hydrochloric acid. This method will be referred to as the *combined method.*

The second method was carried out in the following way. One part of the substance and fifteen parts by weight of 3 per cent sulphuric acid were heated in sealed tubes for 24 hours at a temperature of 100–105°. From time to time the tubes were agitated. On cooling, the fatty acids and sphingosine sulphate separated as a soft cake which could be readily removed by filtration and washed.

Separation of the Product of Hydrolysis into Three Fractions.

Choline Fraction.—This was contained in the aqueous part of the products of hydrolysis. The analysis of this fraction was carried out in the manner described in the previous paper.

Acid Fraction.—In all experiments the acids were separated from the bases by virtue of the nearly complete insolubility of the barium salts of the higher fatty acids in acetone. The free bases, on the other hand, are soluble in the same solvent.

Sphingosine Fraction.—This was present in the acetone filtrate from the soaps.

For convenience of discussion of the results obtained in this work the mode of procedure in the two types of hydrolysis will be reiterated.

Combined Hydrolysis.—The water-insoluble part of the products of hydrolysis was dissolved in a minimum volume of methyl alcohol to which was added in excess a solution of barium hydroxide in methyl alcohol; to complete the separation of the barium soaps an excess of acetone was added. The precipitate consisted of the soaps, and the filtrate contained the ethyl esters of the fatty acids and the water-insoluble bases.

In order to saponify the esters the acetone solution was concentrated under diminished pressure and the residue dissolved in methyl alcohol. To this solution, barium hydroxide in methyl alcohol was added in excess and the product heated on the water bath with a reflux condenser for 6 hours. From the reaction product the separation of the barium soaps was completed by the addition of a considerable quantity of acetone. To further purify the acid fraction the acids were liberated from the barium soaps, and reconverted into the barium soaps by the barium and acetone procedure.

In order to purify the basic fraction the acetone solution was concentrated *in vacuo*, the residue dissolved in methyl alcohol, and the barium hydroxide and acetone procedure was repeated. As a rule, the entire process was repeated three times. The careful treatment of this fraction was found essential. The presence of impurities, particularly of inorganic nature, interferes considerably with the subsequent reduction of the bases with hydrogen.

Sulphuric Acid Hydrolysis.—The water-insoluble products of hydrolysis under these conditions consisted only of the acids and of sphingosine; there was no need for the stage which aimed to saponify the esters. Hence the reaction product was dissolved in methyl alcohol and treated with barium hydroxide and acetone, as described above.

Quantitative Hydrolysis.—These experiments aimed to determine the proportion of the constituents, and their general elementary composition, without attempting to determine the exact nature of the acids and the bases. Their purpose was to ascertain whether the substances isolated in the course of this work make up the entire molecule of sphingomyelin. This hydrolysis was carried out by means of 3 per cent sulphuric acid. 2 gm. of the substance were used for each experiment.

Analysis of Fatty Acids.

Liberation and Purification of Fatty Acids.—The mode of liberation of the fatty acids and of their purification remained the same in all experiments. The barium soaps were suspended in aqueous 10 per cent hydrochloric acid, and allowed to stand on the water bath over night. On cooling, a solid mass formed consisting of the free acids and some adhering barium chloride. The mass was filtered and taken up in hot acetone, which separated the acids from the mineral impurities. The acetone solution was then concentrated *in vacuo* and the residue was again taken up in acetone. The operation was repeated until the residue completely dissolved in acetone, leaving no filterable residue. The acetone was then removed by distillation and the residue concentrated on the water bath to constant weight.

The fatty acids obtained in this manner served for the determination of the elementary composition and of the molecular weight of the mixed acids. Whenever there was still present a slight mineral impurity, the acids were further purified by transferring them into lead salts. The mode of procedure was the following: The acids were dissolved as rapidly as possible in hot methyl alcohol, and the solution was treated with a slight excess of lead acetate dissolved in methyl alcohol. The addition of a few drops of ammonia completed the precipitation. The lead salts were washed with water and with acetone, and then suspended in ether and allowed to stand over night. Finally the lead salts were dissolved in hot toluene and decomposed by means of hydrogen sulphide.

Practically all samples of which the molecular weights are recorded were prepared by this procedure.

Separation of the Fatty Acids.—A general method for the separation of fatty acids of sphingomyelin has as yet not been devised. Of the many employed in course of the present work only those will be reported which helped to bring out two points: first, that one of the acids making up the molecule of the phosphatide is lignoceric; and second, that the other is of a lower molecular weight and appears to be a hydroxy acid.

Experiment I.—90.0 gm. of sphingomyelin were hydrolyzed by the combined method. The acids were isolated in two fractions: the first as the barium salts of the unesterified acids; and the second as the barium salts of the saponified esters.

Analysis of the purified acids of the first fraction:
0.1000 gm. substance gave 0.2769 gm. CO_2 and 0.1144 gm. H_2O.
1.0000 "   "   required for neutralization 6.4 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_3$:	Found:
C...	78.20	75.49
H...	13.20	12.71
Mol. wt.......................................	368	312

Acids of the second fraction:
0.1000 gm. substance gave 0.2772 gm. CO_2 and 0.1158 gm. H_2O.
1.0000 "   "   required for neutralization 6.4 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_3$:	Found:
C...	78.20	75.59
H...	13.20	12.95
Mol. wt.......................................	368	312

The acids were then combined and repeatedly recrystallized at 25°C. out of a large volume of acetone. Finally the most insoluble fraction had a melting point of 80–82°.[5]

1.0000 gm. of this substance required for neutralization 5.6 cc. $\frac{N}{2}$ alkali; hence mol. wt. = 358.

Experiment II.—30.0 gm. substance were hydrolyzed in sealed tubes. The fatty acids were immediately transformed into the methyl esters. The esters were distilled under a pressure of 0.3 mm. Two fractions were obtained, the first distilling up to 155°, the second distilling up to 200°.

[5] All melting points were determined under exactly the same condition; namely, in a sulphuric acid bath provided with a stirring arrangement. The rate of heating was 6 to 7 seconds per 1°C.

The esters of the second fraction had a melting point of 56–57°C., and the following composition:

0.1010 gm. substance gave 0.2906 gm. CO_2 and 0.1190 gm. H_2O.

	Calculated for $C_{26}H_{47}O_2CH_3$:	Found:
C...	78.53	78.47
H...	13.00	13.19

The ester was saponified and the resulting acid had a melting point of 81°C.

Experiment III.—100.0 gm. of sphingomyelin were decomposed by the combined method. The acids were purified by the lead method and fractionated from acetone. The fraction of higher solubility had the following composition:

0.1025 gm. substance gave 0.2758 gm. CO_2 and 0.1096 gm. H_2O.
C = 73.46; H = 11.98.

The substance, again recrystallized out of acetone, had the following composition:

0.1054 gm. substance gave 0.2778 gm. CO_2 and 0.1166 gm. H_2O.
0.3000 " " required 2.0 cc. $\frac{N}{2}$ alkali for neutralization.
The substance had a melting point of 68–69°C.

	Calculated for $C_{18}H_{36}O_3$:	Found:
C...	71.92	71.89
H...	12.10	12.38
Mol. wt....................................	300	300

The substance was converted into the ethyl ester and the ester recrystallized out of alcohol. The middle fraction had the following composition:

0.0844 gm. substance gave 0.2246 gm. CO_2 and 0.0916 gm. H_2O.

	Calculated for $C_{18}H_{35}O_3C_2H_5$:	Found:
C...	73.07	72.58
H...	12.27	12.15

This acid possessed the elementary composition and the molecular weight of hydroxystearic acid. However, the low melting point of the acid warned us against hasty conclusions. A corresponding fraction from the kidney sphingomyelin possessed a still lower molecular weight. Hence decision as to the actual composition of the second acid will be deferred until we accumulate more material which will permit further purification of the acid.

Bases.

The mode of procedure in the analysis of the sphingosine fraction differed in the earlier and later experiments. Originally the fraction was treated with alcoholic sulphuric acid to form the sulphates of the bases. These were then reduced with hydrogen gas in the presence of colloidal palladium after Paal. In later experiments it was found more advantageous to reduce the entire fraction prior to the formation of the sulphates. In order to facilitate reduction, the bases were dissolved in ether and washed with water in a separatory funnel. To the ethereal solution of the bases some acetic acid was added and the solution then reduced with hydrogen.

In order to remove the palladium completely the product of reduction was warmed on the water bath with an equal volume of acetone. The filtrate was concentrated to remove all the acetone and acetic acid. The residue was then dissolved in an equal volume of alcohol and an alcoholic solution of sulphuric acid was added until the mixture reacted faintly acid to Congo red. Care must be taken not to overadd sulphuric acid, as the sulphate is quite soluble in an excess of the acid.

Analysis of the bases obtained in this manner gave the following results:

  I. Combined method of hydrolysis.
     0.1024 gm. substance gave 0.2772 gm. CO_2 and 0.0954 gm. H_2O.
     C = 62.37; H = 10.35.
 II. Combined method of hydrolysis.
     0.1012 gm. substance gave 0.2336 gm. CO_2 and 0.0970 gm. H_2O.
     C = 62.79; H = 10.80.

A great many samples of similar composition were analyzed, and material of such composition served for the isolation of hydroxyheptadecylamine.

Hydroxyheptadecylamine (Sphingine),[6] $C_{17}H_{34}(OH)NH_2$.—This base was obtained by fractional crystallization of the mixed sulphates from a solution of chloroform in amyl alcohol. The first recrystallizations were carried out from a solution in one part of chloroform and three parts of alcohol. The concentration of the chloroform was progressively increased and the final recrystallizations were made

[6] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1916, xxiv, 67.

out of equal parts of the solvents, first at room temperature (20–25°C.) and finally at 35°.

The material obtained in this manner consisted of microscopic prismatic needles, and had the macroscopic appearance of glistening scales.

The composition of this crystalline sulphate was not constant for individual samples, nor for successive recrystallizations of the same sample, although a sufficient number gave on analysis values corresponding to the neutral sulphate of sphingine.

I. 0.1020 gm. substance gave 0.2326 gm. CO_2 and 0.1034 gm. H_2O.
     0.2000 "   "   required for neutralization 5.8 cc. $\frac{N}{10}$ acid.
     0.1000 "   "   gave 0.0366 gm. $BaSO_4$.
II. 0.1012 "   "   " 0.2344 gm. CO_2 and 0.0990 gm. H_2O.
     0.2000 "   "   required for neutralization 5.3 cc. $\frac{N}{10}$ acid.
     0.3000 "   "   gave 0.1126 gm. $BaSO_4$.
III. 0.1020 "   "   " 0.2376 gm. CO_2 and 0.1116 gm. H_2O.

	Calculated for $[C_{17}H_{37}NO]_2H_2SO_4$:	I.	II.	III.
C............................	63.80	63.43	63.43	63.77
H............................	11.85	11.57	11.01	12.10
N............................	4.37	4.06	4.06	
S............................	5.00	5.03	5.15	

These samples on further recrystallization gave values for carbon varying from 64.5 to 66.0, and for hydrogen from 11.5 to 12.5.   However, when converted into the free base they generally had the uniform composition of sphingine.

Free Sphingine.—For the preparation of the free base the sulphate was treated in the same manner as sphingosine sulphate.   The base crystallized out of a minimal quantity of petrolic ether in the form of plates resembling cholesterol.   The substance had a melting point of 83.5°, and had the following composition:

0.1046 gm. substance gave 0.2844 gm. CO_2 and 0.1280 gm. H_2O.
0.1056 "   "   " 0.2868 "   "   " 0.1238 "   "

	Calculated for $C_{17}H_{37}NO$:		Found:	
C....................................	75.30		74.90	75.35
H....................................	13.65		13.82	13.35

In a 10 per cent solution of sulphuric acid in alcohol the substance had the following rotation:

$$[\alpha]_D^{25} = \frac{-0.34° \times 1.7660}{1 \quad \times 0.1000} = -6.00°$$

Diacetylhydroxyheptadecylamine.—0.6000 gm. of the free base was heated with acetic anhydride for 1 hour under a reflux condenser. On cooling the acetyl derivative crystallized in the form of long needles. The substance was recrystallized out of acetone. It melts at 109.5°C. (corrected).

0.1050 gm. substance gave 0.2730 gm. CO_2 and 0.1108 gm. H_2O.
0.1000 " " required for neutralization 2.71 $\frac{N}{10}$ cc. acid.

	Calculated for $C_{17}H_{33}NO(CH_3CO)_2$:	Found:
C...	70.90	70.90
H...	11.55	11.80
N...	3.97	3.79

In a solution in equal parts of chloroform and methyl alcohol the substance gave the following rotation:

$$[\alpha]_D^{25} = \frac{+0.44° \times 2.3234}{1 \quad \times 0.0500} = +20.44°$$

Dihydrosphingosine (*Dihydroxyheptadecylamine*).—For the identification of sphingosine, use was made of the hydrolysis by means of 3 per cent aqueous sulphuric acid. The basic fraction was reduced with hydrogen in the presence of colloidal palladium. Since the purification by means of repeated recrystallization out of alcohol containing small quantities of sulphuric acid gave unsatisfactory results, it was considered essential to analyze the free base.

Analysis of the sulphate:

0.1040 gm. substance gave 0.2324 gm. CO_2 and 0.1070 gm. H_2O.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$	Found:
C...	60.61	60.94
H...	11.38	11.51

The free base was obtained by the process employed in course of this work.

0.1023 gm. substance gave 0.2664 gm. CO_2 and 0.1204 gm. H_2O.

	Calculated for $C_{17}H_{37}NO_2$:	Found:
C...	71.07	71.02
H...	12.89	13.17

Hydroxyheptadecylamine from Cerebrin.—80.0 gm. of cerebrin, corresponding to Thierfelder's cerebron, were hydrolyzed by the

combined method, and the mode of procedure employed in the hydrolysis of sphingomyelin was followed closely. The free base obtained in this manner had the physical properties of sphingine and the following composition:

0.1042 gm. substance gave 0.2874 gm. CO_2 and 0.1232 gm. H_2O.

	Calculated for $C_{17}H_{37}NO$:	Found:
C..	75.30	75.12
H..	13.65	13.23

Choline.—Choline was isolated from the aqueous part of the product of hydrolysis. When the hydrolysis was accomplished by means of aqueous sulphuric acid, the aqueous filtrate was treated with barium carbonate and the filtrate from barium sulphate was concentrated to dryness under diminished pressure. The residue was taken up in alcohol, filtered, and concentrated, and the operation was repeated several times. The final product was acidulated with hydrochloric acid and treated with an alcoholic solution of platinic chloride. The platinic salt was recrystallized out of water.

When barium hydroxide was used for hydrolysis the aqueous part of the product of hydrolysis was saturated with carbon dioxide gas, and the filtrate from barium carbonate was used for the preparation of the chloroplatinic salt of choline. The solution apparently contained no other bases than choline, since on treatment with nitrous acid in Van Slyke's apparatus it did not give off nitrogen gas.

0.1018 gm. substance gave 0.0732 gm. CO_2, 0.0430 gm. H_2O, and 0.0316 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C..	19.48	19.60
H..	4.58	4.69
Pt..	31.65	31.05

Quantitative Hydrolysis.

In order to obtain an estimate of the fatty acids and of sphingosine in sphingomyelin, hydrolysis was carried out on 2.0 gm. of the substance in a sealed tube with a 3 per cent sulphuric acid solution. The mode of procedure was the same as that carried out on larger quantities of material.

The empirical data obtained in this work will be compared with the theoretical for the monophosphatide containing lignoceric acid in its molecule.

	Calculated for $C_{46}H_{91}N_2O_7P$:	Found:
Sphingosine	35.56	34.10
Acids	40.58	43.00

The crude sphingosine fraction contained 4.5 per cent nitrogen. It was transformed into a sulphate of the following composition:

0.1012 gm. substance gave 0.2218 gm. CO_2 and 0.0928 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_3)_2H_2SO_4$:	Found:
C	61.08	60.97
H	10.78	10.47

Analysis of the acid fraction:

0.1022 gm. substance gave 0.2876 gm. CO_2 and 0.1188 gm. H_2O.

1.0000 " " was titrated, with $\frac{N}{2}$ alkali and required for neutralization 6.3 cc.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	76.75
H	13.20	13.00
Mol. wt.	368	317

II. Beef Kidney Sphingomyelin.

A phosphatide of this group was first discovered in the extracts from beef kidney by E. K. Dunham,[7] and was named by him carnaubon. Later MacLean[8] questioned the correctness of Dunham's views by showing that carnaubon could be further purified, and still later Rosenheim and MacLean[9] have demonstrated that carnaubon contained an admixture of galactosides, and that the acid assumed by Dunham to be carnaubic had the physical properties of lignoceric acid. However, Rosenheim and MacLean have not isolated the pure sphingomyelin.

The present investigation was in progress at the time of the publication of their results.

[7] Dunham, E. K., Proc. Soc. Exper. Biol. and Med., 1904–05, ii, 63; Jour. Biol. Chem., 1908, iv, 297. Dunham, E. K., and Jacobson, C. A., Z. f. physiol. Chem., 1910, lxiv, 302.

[8] MacLean, H., Biochem. Jour., 1912, vi, 333.

[9] Rosenheim, O., and MacLean, H., Biochem. Jour., 1915, ix, 103.

Preparation and Composition.—All the details found necessary for the preparation of brain sphingomyelin were applied for the isolation of the substance from the kidney tissue. Several samples were analyzed and all seemed to contain a slightly lower proportion of carbon. This was possibly due to the fact that the material from the kidney contained a higher proportion of the phosphatide with the fatty acid of a molecular weight lower than that of lignoceric.

I. 0.1032 gm. substance gave 0.2412 gm. CO_2 and 0.1052 gm. H_2O.
   0.3000 " " required for neutralization 7.5 cc. $\frac{N}{10}$ acid.
   0.4000 " " gave 0.0548 gm. $Mg_2P_2O_7$.
I. 0.0990 " " " 0.2330 gm. CO_2 and 0.1042 gm. H_2O.
   0.2000 " " required for neutralization 4.75 cc. $\frac{N}{10}$ acid.
   0.3000 " " gave 0.0404 gm. $Mg_2P_2O_7$.

	C	H	N	P
I......................................	64.80	11.41	3.50	3.82
II.....................................	64.53	11.83	3.52	3.96

In a solution of methyl alcohol and chloroform Sample I gave the following optical rotation:

$$[\alpha]_D^{26} = \frac{+0.36° \times 3.6510}{0.5 \ \times 0.3000} = +8.73°$$

Quantitative Hydrolysis.

2.0 gm. of Sample I, hydrolyzed for 24 hours in a sealed tube with 3 per cent sulphuric acid, gave 32.1 per cent of its weight in the sphingosine fraction and 49.0 per cent in the fraction of the fatty acid.

Choline Fraction.—This fraction was analyzed in the same manner as the corresponding fraction from brain sphingomyelin. The base was identified as the salt of chloroplatinic acid. The salt was recrystallized out of water.

0.1048 gm. substance gave 0.752 gm. CO_2, 0.0434 gm. H_2O, and 0.0334 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C..	19.48	19.58
H..	4.58	4.63
Pt...	31.65	31.87

Acid Fraction.—Here also final results on the separation of the two acids have not yet been obtained. The mixed acid showed approximately the same composition and molecular weight as the corresponding material from the brain sphingomyelin.

Experiment I.—Acid hydrolysis. The acid fraction was purified through the lead salts.

I. 0.1020 gm. substance gave 0.2830 gm. CO_2 and 0.1135 gm. H_2O.
II. 0.1014 "   "   "   0.2846 "   "   " 0.1128 "   "
   0.4062 " mixed acids required for neutralization 2.56 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_2$:	Found: I.	II.
C...	78.20	75.96	76.53
H...	13.20	12.49	12.45
Mol. wt................................	368	312	315

Experiment II.—The most convenient method for obtaining lignoceric acid was through the process of alcoholysis. 9.0 gm. of sphingomyelin in a solution of 4.0 cc. of sulphuric acid in 200 cc. of methyl alcohol were heated under a reflux condenser on the water bath for 6 hours. On cooling an ester separated out in bright scales. Recrystallized out of acetone, the substance contained: C, 74.11, and H, 12.13. The substance contained 1.17 per cent of ash. It was distilled at a pressure of 0.4 mm. The temperature of distillation was allowed to rise to 200°C. The distillate was recrystallized out of acetone. The substance had a melting point of 56–57° and the following composition:

0.1020 gm. substance gave 0.2934 gm. CO_2 and 0.1200 gm. H_2O.

	Calculated for $C_{24}H_{47}O_2CH_3$:	Found:
C...	78.53	78.44
H...	13.09	13.17

The acid of the lower molecular weight has not yet been isolated in a sufficient degree of purity. The best results were obtained by fractionation from acetone. In two experiments a substance was obtained of apparently the same composition and molecular weight. But here again decision regarding its true composition will have to be deferred until a larger quantity of the acid is on hand. In both experiments the acids were purified through the lead salts prior to fractionation. The two results will be described under Experiment III.

Experiment III.—The mixed acids were recrystallized out of acetone at 0°, the mother liquor was concentrated and again taken up in hot acetone. The final substance had the following composition:

0.0952 gm. substance gave 0.2558 gm. CO_2 and 0.1152 gm. H_2O.
C = 69.76; H = 10.65.

A second sample, obtained in the same manner, had the following composition:

0.1042 gm. substance gave 0.2670 gm. CO_2 and 0.1032 gm. H_2O.
C = 69.88; H = 11.09.

Sphingosine Fraction.—The mixed bases obtained by the combined method of hydrolysis, following exactly the mode of procedure described above, gave results approximating those obtained on the brain sphingomyelin. The product consisted of long needles.

0.1046 gm. substance gave 0.2400 gm. CO_2 and 0.1092 gm. H_2O.
C = 62.79; H = 11.69.

This substance was further fractionated from amyl alcohol and chloroform and finally transformed into the free base. This had the appearance of sphingine and the following composition:

0.1030 gm. substance gave 0.2756 gm. CO_2 and 0.1222 gm. H_2O.

	Calculated for $C_{17}H_{37}NO$:	Calculated for $C_{17}H_{37}NO_3$:	Found:
C...............................	75.30	71.07	73.12
H...............................	13.65	12.89	13.30

Pure sphingosine was more accessible through the sulphuric acid hydrolysis. The substance was reduced with hydrogen prior to conversion into the sulphate.

I. 0.0992 gm. substance gave 0.2158 gm. CO_2 and 0.1042 gm. H_2O.
  0.1410 "   " required for neutralization 4.25 cc. $\frac{N}{10}$ acid.
II. 0.1048 "   " gave 0.2340 gm. CO_2 and 0.1032 gm. H_2O.
  0.1500 "   " required for neutralization 4.34 cc. $\frac{N}{10}$ acid.

	Calculated for $(C_{17}H_{37}NO_3)_2H_2SO_4$:	Found: I.	II.
C...............................	60.61	60.67	60.90
H...............................	11.38	11.35	11.02
N...............................	4.16	4.23	4.05

Lignoceryl Sphingine.

The substance was obtained in the course of a combined hydrolysis of 100 gm. of Sample I (page 374). The basic fraction was reduced with hydrogen in the presence of palladium after Paal. The reaction product was warmed on the water bath and filtered. The filtrate

was concentrated nearly to dryness on the water bath. The residue was dissolved in 400 cc. of hot alcohol and filtered hot. While the solution was still warm a crystalline precipitate formed. This was immediately filtered, and recrystallized out of alcohol. It crystallized in the form of rosettes composed of needles.

The substance gave no nitrogen gas on treatment with nitrous acid according to Van Slyke.

0.1018 gm. substance gave 0.2874 gm. CO_2 and 0.1173 gm. H_2O.
0.1473 " " neutralized 2.72 cc. $\frac{N}{10}$ acid.

	Calculated for $C_{17}H_{34}(OH)NH.C_{24}H_{47}O$:	Found:
C..	79.39	79.60
H..	13.36	13.32
N..	2.26	2.59

Lack of material prevented further work on this substance.

III. Liver Sphingomyelin.

No phosphatide of this type has been previously obtained from this organ. The substance here analyzed was prepared in the usual way. The crude sphingomyelin required a great many recrystallizations from pyridine and chloroform. Finally it reacted towards orcin in the same manner as sphingomyelin of other origin.

0.1067 gm. substance gave 0.2500 gm. CO_2 and 0.1088 gm. H_2O.
0.3000 " " required for neutralization 7.3 cc. $\frac{N}{10}$ acid.
0.4000 " " gave 0.0548 gm. $Mg_2P_2O_7$.

C	H	N	P
64.47	11.57	3.41	3.81

In a solution of equal parts of methyl alcohol and chloroform the substance had the following rotation:

$$[\alpha]_D^{32} = \frac{+0.31° \times 3.6490}{0.5 \times 0.3000} = +7.61°$$

Quantitative Hydrolysis.

2.0 gm. substance, hydrolyzed with 3 per cent H_2SO_4, gave 32.14 per cent of its weight in the form of sphingosine and 41.70 per cent in the form of acids.

	Calculated for $C_{48}H_{95}N_2O_7P$:	Found:
Sphingosine	35.56	32.14
Acids..	40.58	41.70

Choline.—This base was obtained in the usual way. The salt of chloroplatinic acid was recrystallized out of water.

0.1000 gm. substance gave 0.0718 gm. CO_2, 0.0428 gm. H_2O, and 0.0312 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.58
H	4.58	4.79
Pt	31.65	31.20

Sphingosine.—The substance was obtained in the usual way. It was analyzed in the form of the sulphate.

0.1075 gm. substance gave 0.2282 gm. CO_2 and 0.0952 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_2)_2H_2SO_4$:	Found:
C	61.08	60.78
H	10.78	· 10.40

Acids.—The mixed acids were obtained by hydrolysis with 3 per cent sulphuric acid in sealed tubes and had the following composition:

0.1075 gm. substance gave 0.2956 gm. CO_2 and 0.1206 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.00
H	13.20	12.60

IV. Egg Yolk Sphingomyelin.

A phosphatide approximating in composition that of Thudichum's sphingomyelin was described by Stern and Thierfelder.[10] The material described here was prepared by the same mode of procedure used in the work on sphingomyelin from other sources and had the same microscopic appearance. It gave a negative test on boiling with orcin in the presence of copper salts.

0.0993 gm. substance gave 0.2354 gm. CO_2 and 0.1030 gm. H_2O.
0.4000 "   "   "   0.0606 gm. $Mg_2P_2O_7$.
0.3000 "   "   required for neutralization 8.3 cc. $\frac{N}{10}$ acid.

C	H	N	P
65.00	11.68	3.84	4.22

In a solution of equal parts of methyl alcohol and chloroform the substance had the following rotation:

$$[\alpha]_D^{25} = \frac{+0.31° \times 3.6500}{0.5 \ \times 0.3000} = +7.54°$$

[10] Stern, M., and Thierfelder, H., *Z. f. physiol. Chem.*, 1907, liii, 370.

Quantitative Hydrolysis.

2.0 gm. substance, hydrolyzed with 3 per cent sulphuric acid, gave in the acid fraction 43.14 per cent of its weight and 33.70 per cent in the sphingosine fraction.

	Calculated for $C_{44}H_{88}N_2O_7P$:	Found:
Sphingosine	35.56	33.70
Acids	40.58	43.14

Choline.—This was obtained in the usual way and recrystallized out of water.

0.1030 gm. substance gave 0.0734 gm. CO_2, 0.0460 gm. H_2O, and 0.0324 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.43
H	4.58	4.88
Pt	31.65	31.46

Sphingosine Fraction.—This was obtained in the usual way and reduced to dihydrosphingosine. The value obtained for the carbon slightly exceeds the theoretical requirement, and the evidence is accepted as satisfactory only in the light of the experience gained with the substance obtained from the brain and from the kidneys.

0.1039 gm. substance gave 0.2348 gm. CO_2 and 0.1054 gm. H_2O.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found:
C	60.61	61.75
H	11.38	11.37

Analysis of Mixed Acids.—The acids were obtained by hydrolysis with 3 per cent sulphuric acid and had the same composition as the acids obtained from sphingomyelin originating from other sources.

0.1070 gm. substance gave 0.2914 gm. CO_2 and 0.1266 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.29
H	13.20	12.24

CEPHALIN.[1]

III. CEPHALIN OF THE EGG YOLK, KIDNEY, AND LIVER.

PRELIMINARY PAPER.

BY P. A. LEVENE AND C. J. WEST.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The principal object of the present investigation was the search for a convenient source of cephalin. It is true that the brain tissue contains a high proportion of this substance, but it also contains a variety of other phosphatides. Experience has shown that because of this the purification of cephalin from the brain tissue met with great obstacles. It was hoped that in some other organ the distribution of phosphatides might be so much in favor of cephalin that it could be readily freed from impurities. Unfortunately this expectation was not realized for the present. The brain still remains the most convenient source of cephalin. On the other hand, the results of the present work have shown that the cephalins obtained from various organs and from egg yolk possess identical properties and composition, and hence the results of the structural analysis of one will apply to all.

The following table contains the results of the ultimate analysis of cephalin prepared by different workers.

Author.	Source.	C	H	N	P
Levene and West[1]	Brain	60.49	9.52	1.96	3.80
Stern and Thierfelder[2]	Egg yolk	59.68	9.74	1.57	3.64
Levene and West	"	60.00	9.62	1.78	3.69
Frank[3]	Liver	57.10	9.62	1.72	3.91
Levene and West	"	60.33	9.01	1.86	3.75
" " "	Kidney	60.17	8.95	1.70	3.65

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913–14, xvi, 419; 1916, xxiv, 41.

[2] Stern, M., and Thierfelder, H., *Z. f. physiol. Chem.*, 1907, liii, 379.

[3] Frank, A., *Biochem. Z.*, 1913, 1, 277.

From the table it is seen that from egg yolk the material obtained by us did not differ from that prepared by Stern and Thierfelder, although we employed a more rigid procedure of purification. On the other hand, the substance isolated by us from the liver was undoubtedly in a higher state of purity than that described by Frank, and finally in regard to the kidney the present is the first report of the isolation of cephalin from that organ.

Work on other organs is in progress.

<center>EXPERIMENTAL PART.</center>

Egg Yolk Cephalin.

Earlier workers and many of the later workers in lipoid chemistry considered all of the lipoid fraction of egg yolk as lecithin.[4] Erlandsen[5] was the first to question the composition of the lecithin fraction. During the course of his work on the lipoids of heart muscle, he attempted to isolate cuorin from egg yolk, but was unsuccessful. In the same year Stern and Thierfelder[2] reported the isolation of an alcohol-insoluble fraction from the so called lecithin. The compound obtained from fresh eggs resembled cephalin in its properties, and analyzed for the theoretical cephalin composed of stearic and cephalinic acids, glycerophosphoric acid, and amino-ethyl alcohol.[6] The cephalin from a commercial egg preparation, however, showed the composition found by other authors; namely, C, 59.68; H, 9.74; N, 1.57; P, 3.64. The only explanation offered for the different results was that possibly the age of the egg had some effect. We have repeated the work on the egg yolk, using commercial egg yolk powder.

Alcohol Extraction.

Egg yolk powder was extracted four or five times with hot alcohol, the alcoholic extract concentrated and cooled at 0°. The egg oil and

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[4] For example, Serono, C., and Palozzi, A. (*Arch. farm. sperim.*, 1911, xi, 553), state that 11–12 per cent of the egg yolk is lecithin, but mention no other lipoid. It is interesting in this connection to compare the work of Barbieri, N. A. (*Compt. rend. Acad. d. sc.*, 1910, cli, 405), who denies the existence of free or bound lecithin in the egg yolk.

[5] Erlandsen, A., *Z. f. physiol. Chem.*, 1907, li, 150.

[6] See Levene and West, *Jour. Biol. Chem.*, 1916, xxiv, 41.

lipoids separated as a semisolid mass. Since it was impossible to filter this, even at 0°, the entire product was stirred with a large quantity of dry acetone. This dissolved the egg oil and considerable quantities of lipoid. The acetone extract was concentrated and again extracted with dry acetone, and the process repeated until the oil dissolved completely in the acetone. All the lipoid fractions were then extracted with ether, and the small amount of sphingomyelin was removed by filtration or centrifugation. The ether-soluble fraction was then separated by precipitation with absolute alcohol into the cephalin (alcohol-insoluble) and lecithin (alcohol-soluble) fractions.

This crude cephalin fraction contained 1.8 per cent nitrogen. When treated with alcohol at 60–70°, as recommended by MacLean for the preparation of cuorin, a product was obtained with the same composition:

0.500 gm. substance neutralized 6.18 cc. 0.1 N acid.
0.300 "   "   gave 0.0390 gm. $Mg_2P_2O_7$.

	N	P
Found	1.72	3.62

The material was then precipitated from alcohol at 60°, in the same manner as described for the purification of brain cephalin. The two fractions (I, insoluble, and II, soluble) showed the same composition:

I. 0.500 gm. substance neutralized 6.34 cc. 0.1 N acid.
    0.300 "   "   gave 0.0386 gm. $Mg_2P_2O_7$.
II. 0.500 "   "   neutralized 6.48 cc. 0.1 N acid.
    0.300 "   "   gave 0.0404 gm. $Mg_2P_2O_7$.

	Found:	
	N	P
I	1.75	3.60
II	1.81	3.74

Finally, the ether solution was shaken with dilute hydrochloric acid and the resulting solution repeatedly precipitated with acetone.

0.500 gm. substance neutralized 6.33 cc. 0.1 N acid.
0.300 "   "   gave 0.0398 gm. $Mg_2P_2O_7$.
0.1484 "   "   " 0.3265 " CO_2 and 0.1276 gm. H_2O.
0.300 "   "   " 0.0662 " AgI (glycerol determination).

	C	H	N	P	Glycerol.
Found	60.00	9.62	1.78	3.69	8.65

Ether Extraction.

In order to determine whether the initial solvent used had any effect upon the composition of the alcohol-insoluble fraction, a second lot of egg powder was extracted repeatedly with ether at room temperature. The combined ether extract was treated as given above. The fraction, insoluble in alcohol at 60°, had the following composition:

0.500 gm. substance neutralized 6.53 cc. 0.1 N acid.
0.300 "   "   gave 0.0400 gm.$Mg_2P_2O_7$.
0.1516 "   "   "   0.3327 " CO_2 and 0.1273 gm. H_2O.
0.300 "   "   "   0.0660 " AgI (glycerol determination).

	C	H	N	P	Glycerol.
Found	59.86	9.40	1.82	3.70	8.65

Commercial Lecithin.

The same cephalin has also been obtained from a sample of commercial lecithin. This was found to consist of considerable egg oil, together with sphingomyelin, cephalin, and lecithin. The cephalin fraction, insoluble in alcohol at 60°, had the same composition as the two samples analyzed above, and figures need not be given.

Lead Salt.

The lead salt was prepared by the method given in our second paper and had the following composition:

0.500 gm. substance neutralized 3.01 cc. 0.1 N acid.
0.300 "   "   gave 0.0262 gm. $Mg_2P_2O_7$.

	N	P
Found	1.05	2.43

Kidney Cephalin.

A review of the work on the lipoids of the kidney has been given by Forbes and Keith.[7] The most important and complete investigation is that of MacLean.[8] In the present work cephalin was obtained

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[7] Forbes, E. B., and Keith, M. H., Phosphorus Compounds in Animal Metabolism, Wooster, 1914, 141.

[8] MacLean, H., *Biochem. Jour.*, 1912, vi, 333.

from the kidney by the same method as that used in the work on brain cephalin. The product insoluble in alcohol at 60° had the following composition:

0.500 gm. substance neutralized 6.08 cc. 0.1 N acid.
0.300 "   "   gave 0.0394 gm. $Mg_2P_2O_7$.
0.1050"   "   "   0.2316 " CO_2 and 0.0840 gm. H_2O.

	C	H	N	P
Found..	60.17	8.95	1.70	3.65

The same results were obtained when the dried kidney material was extracted with ether. Because of the difficulty of purifying cephalin in general, no other details of the work will be given here.

Liver Cephalin.

The first alcohol-insoluble lipoid isolated from the liver was called jecorin.[9] The widely divergent results obtained by various observers indicated clearly that this substance was a mixture.[10] Baskoff[11] obtained a cuorin-like substance which he called *heparphosphatide;* this had a N : P ratio of about 1 : 1.5. Still later Frank[10] obtained a compound which resembled cephalin in its composition and properties.

The liver was worked up in the same general way as the other organs. The ether-soluble material of the alcoholic extract was precipitated repeatedly with alcohol and then with acetone. The cephalin was then emulsified with water, precipitated with 10 per cent hydrochloric acid, the precipitate taken up in ether and repeatedly precipitated with dry acetone. Solutions in amyl alcohol or ethyl

[9] Drechsel. E., *Jour. f. prakt. Chem.*, 1886, xxxiii, 425. Baldi, D., *Arch. Physiol.*, Suppl., 1887, 100. Jacobsen, A., *Skand. Arch. f. Physiol.*, 1895, vi, 262. Manasse, P., *Z. f. physiol. Chem.*, 1895, xx, 478. Drechsel, *Z. f. Biol.*, 1896, xxxiii, 86. Henriques, V., *Z. f. physiol. Chem.*, 1897, xxiii, 244. Meinertz, J., *Z. f. physiol. Chem.*, 1905, xliv, 371. Siegfried, M., and Mark, H., *Z. f. physiol. Chem.*, 1905, xlvi, 492. Waldvogel and Tintemann, *Z. f. physiol. Chem.*, 1906, xlvii, 129. Mayer, P., *Biochem. Z.*, 1906, i, 81. Baskoff, A., *Z. f. physiol. Chem.*, 1908, lvii, 395; 1909, lxi, 426; 1909, lxii, 162.

[10] For table of analyses and discussion, see Frank, A., *Biochem. Z.*, 1913, l, 277.

[11] Baskoff, *Z. f. physiol. Chem.*, 1908, lvii, 395.

acetate deposited the cephalin upon cooling, without any purification (see Baskoff). The material had the following composition:

0.500 gm. substance neutralized 6.8 cc. 0.1 N acid.
0.300 "   "   gave 0.0404 gm. $Mg_2P_2O_7$.
0.1622"   "   " 0.3789 " CO_2 and 0.1306 gm. H_2O.

	C	H	N	P
Found	60.33	9.01	1.86	3.75

This material thus has the composition of cephalin. However when fractionation out of alcohol at 60° was tried, it was found that this material could be separated into two rather constant fractions. The alcohol-soluble fraction had the composition of cephalin, while the alcohol-insoluble fraction contained a higher nitrogen content. In one case we were able to obtain a N: P ratio of nearly '2: 1. We are not prepared, as yet, to discuss the significance of this fraction.

The so called cephalin, obtained above, was dissolved in ether and poured, with stirring, into alcohol heated to 60°. The filtrate was concentrated, and the material again precipitated in the same way. The third precipitation gives a body with the same N: P ratio, both in the precipitate and in the filtrate. This substance has all the properties of cephalin.

0.500 gm. substance neutralized 5.9 cc. 0.1 N acid.
0.300 "   "   gave 0.0394 gm. $Mg_2P_2O_7$.
0.1532"   "   " 0.3404 " CO_2 and 0.1260 gm. H_2O.

	C	H	N	P
Found	60.6	9.20	1.65	3.66

Lead Salt.

The lead salt was prepared as usual. The first analysis is of a sample which had been washed with acetone; the second, of a sample which had been extracted with boiling methyl alcohol.

I. 0.500 gm. substance neutralized 3.96 cc. 0.1 N acid.
   0.300 "   "   gave 0.0250 gm. $Mg_2P_2O_7$.
II. 0.500 "   "   neutralized 3.77 cc. 0.1 N acid.
   0.300 "   "   gave 0.0282 gm. $Mg_2P_2O_7$.

	Found:	
	N	P
I	1.10	2.32
II	1.06	2.61

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

I. THE PROBLEM OF THE CHEMOTHERAPY OF EXPERIMENTAL BACTERIAL INFECTIONS.

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(Received for publication, March 1, 1916.)

The researches of Ehrlich and his coworkers during the past decade, which led to the discovery of salvarsan, have created a new procedure in the search for remedies for the control of infectious diseases. The discovery of this drug was the result of a logically conducted development of the chemistry of organic arsenic compounds in the directions indicated by their biological properties. This new procedure may be regarded in a way as a revival of the one in use during the time preceding the period of purely biological method, for at that time the therapeutic values of quinine, mercury, arsenic, and salicylic acid were first discovered. The resemblance of the old to the new consists essentially in the fact that in each case chemical substances were used as the therapeutic agents, with the difference, however, that the finding of the old chemical remedies in no way involved the use of synthetic organic chemistry, controlled by constant biological cooperation, the essential feature of the new chemotherapeutic method. This new departure must not be confused with the older synthetic pharmacology, the aim of which was quite different.

A few workers, and the number is constantly increasing, have been attracted to the new field, and the occasional report of promising results, taken with the drugs already proven of therapeutic value, affords ample justification for greater expectations. But to each worker the first and perhaps greatest problem which presents itself is the finding of leads. Salvarsan, optochin, and the trypan dyes, the chief products of the new chemotherapy, all owe their discovery to the development of leads which had been furnished by clinical observations in the

field. Arsenic, mercury, quinine, and certain dyes are perhaps the only substances which up to now have been regarded with assurance as leads for chemotherapeutic development. And of these, with the exception of optochin and perhaps of salicylic acid, all have been indicated for protozoan infections only, and there is promise that by the proper development of these substances the problem of the control of most protozoan infections will be successfully solved.

When we turn, however, to the cure of infections of purely bacterial origin the problem which confronts us is found to be more difficult of attack. Here not only the difficulty of procuring a suitable experimental infection as a test object, but the absence of clinical observations which might be regarded in the nature of leads which could form the basis for a rational chemotherapeutic procedure, makes it imperative from the start to seek for such leads. The successful chemical development of quinine which led to the discovery of ethylhydrocuprein already indicates that resource may be had eventually to the above mentioned protozoan leads. And it is a fact that perhaps with the exception of salicylic acid this drug is the first substance to have been used successfully in an experimental bacterial infection. This one fact creates the hope that chemotherapy may find a wider application in the control of bacterial infections. It is conceivable, however, that ultimate success may depend upon the finding of leads other than those which have been successfully used against protozoan infections.

It would seem that a wealth of material should lie ready among the numerous classes of organic substances which have been found to exert powerful bactericidal effects *in vitro*. But the failure of many of these when tried in experimental infections has led to the realization that besides a bacteria-killing property the fulfillment of certain other requirements is essential for the achievement of an internal antisepsis. Some workers believe that no indication of the probable effect of a substance *in vivo* can be discerned from its action *in vitro*, and that the successful control of an infection by chemical agents can be attained only by indirect means. This might occur, on the one hand, as perhaps in the case of atoxyl, by the chemical transformation *in vivo* of the injected drug into some active form. On the other hand, the substance might act through the protective mechanism of

the host either by increasing phagocytosis or by stimulating the production of immunity principles. It is, of course, probable that in some instances these phenomena might play a part and that a possible scheme of chemotherapeutic attack could be developed along these lines.

Owing to the ease of handling bacteria *in vitro* and the simplicity of the bactericidal tests it would be unwise because of former failures to condemn the *in vitro* method, at least as a means of initial orientation. If it were accompanied by certain parallel studies the *in vitro* method should do more than afford only orientating data. As pointed out by Bechhold and Ehrlich[1] and others in the past, besides a mere bactericidal power other conditions must be fulfilled before a substance may be considered even a possibility as a therapeutic agent, provided of course a direct action by the drug itself is in question.

Aside from the obvious conditions of solubility and relative nontoxicity, the drug once in the circulation, whether by direct intravenous injection or by absorption, must be maintained therein for a sufficient length of time and in sufficient concentration in order to unfold its *in vitro* effect. In other words, its free access to the foci of infection should not be completely obstructed.

To accomplish this it must not enter too rapidly into chemical or physical combination with the constituents of the tissues, of which the blood is a fair representative. It must not be too speedily eliminated. And, finally, it must not be too rapidly altered in any way by metabolic processes which would nullify its bactericidal character. There may be still other and less definite factors which separate the *in vitro* from the desired *in vivo* result. If, however, the *in vitro* bactericidal tests could be complemented by a parallel study of those properties of substances which would decide whether they could satisfy the above requirements *in vivo*, our choice of substances for the *in vivo* experiments could be in great measure controlled. A system, though somewhat arbitrary, would be substituted in an undertaking which would otherwise be directed by a haphazard and entirely opportunistic policy.

These considerations have convinced us that the procedure in the search for leads in the chemotherapy of bacterial infections may be

[1] Bechhold, H., and Ehrlich, P., *Ztschr. f. physiol. Chem.*, 1906, xlvii, 173.

logically systematized as follows: Substances which either by their general structure or by the possession of characteristic atomic groups are representative of as many types of organic substances as possible should be systematically selected for bacteriological and biological testing. Such facts as the bactericidal power and partial specificity for certain types, compatibility with tissue constituents (serum), and resistance to profound and rapid metabolic alteration should be noted and considered in the final interpretation of what in the chemical constitution of the substances is responsible for the observed biological behavior.

With organic substances there will be considerable difficulty in satisfying the last requirement. In the case of arsenicals and mer-curials it is immaterial whether metabolization should occur, for the therapeutic characteristics of such compounds are elements. Their value may partly depend upon such metabolization. It does not seem improbable, however, that bactericidal substances may be found which, even though to a less degree than the arsenicals and mercurials, may be sufficiently resistant to metabolic changes to enable them to produce a sterilizing effect before they are disposed of by the host. The large number of pharmacologically active preparations must all persist long enough after administration to produce their physiologi-cal actions.

From the representative substances which have been found to possess the required biological properties, two classes of leads might be obtained: first, those substances which, like quinine, owe their bactericidal action to the general structure of the molecule; and, second, those which, like phenol, are bactericidal principally be-cause of the possession of a certain atomic group. Once in the possession of *bactericidogenic*,[2] tissue-compatible molecules or side-chains, the same systematic development so successfully employed in the development of organic arsenicals by the alteration or addition of groups to the molecule might be here repeated in order to augment the specific bactericidal action, to detoxify it, or in some other way

[2] The word *bactericidogenic*, of obvious derivation, is employed in this and the following articles as a convenient term to express the property of certain chemical groups, when introduced into an organic molecule, of imparting bactericidal prop-erties to that molecule.

furnish it with biologically desirable properties. In this way sub-
stances could be obtained which would form a rational basis for
chemotherapeutic investigations.

The problem of the chemotherapy of bacterial infections and a pos-
sible scheme for its systematic attack have been discussed above in
some detail with the purpose of affording a basis for a better under-
standing of the material which will be presented in the following
papers.

From its nature this material will touch on but one phase of the
above scheme and no claim is made of its complete realization. We
shall present the results obtained in a systematic attempt to alter
chemically the molecule of hexamethylenetetramine with the object
of obtaining a class of bactericidal substances which could be em-
ployed in experimental infections. The use of this drug was inspired
by the interest felt by Dr. Flexner in the possible application of some
of its derivatives in the treatment of experimental poliomyelitis, and
the material which will here be presented is but a part of a larger
undertaking executed with it.

We shall attempt to show how, by the selection of a certain molec-
ular group, namely hexamethylenetetramine, it has been possible to
demonstrate its general bactericidogenic character. By the combi-
nation of this substance in the form of quaternary salts, in the
manner to be described later, with a great variety of other molecular
groupings a new class of bactericidal substances has been prepared[3]
in which the bactericidal nature was principally attributable to the
hexamethylenetetramine nucleus. On the other hand, the degree
of this action was determined by the nature of the molecular
groups added to hexamethylenetetramine. These added groups
were likewise responsible for the partial specificity of certain of the
preparations for particular bacterial species. This partial specificity
did not favor one species alone, but all the species tested were found
to be separately and specifically susceptible to some particular type
of hexamethylenetetramine derivative. We must therefore conclude
that the bactericidogenic character of hexamethylenetetramine ex-

[3] For the chemistry of these substances and the references to those prepared
by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx,
659, 685; 1915, xxi, 103, 145, 403, 439, 455, 465.

hibited in its quaternary salts is not specific but general in character. The specificity, however, is furnished by the proper choice of the molecular grouping added.

It will also be shown that a few of the hexamethylenetetraminium compounds which were tested were either not at all or but slightly inhibited by serum. A few, on the other hand, were found to be greatly inhibited by serum. The fact, however, that any one of the hexamethylenetetraminium salts is compatible with serum is enough to demonstrate the serum compatibility of the bactericidogenic hexamethylenetetramine portion of the molecule itself. We have here, therefore, a bactericidogenic, serum-compatible group. The remainder of the molecule determines the serum incompatibility of those substances the action of which was found to be inhibited by serum.

In the same way the toxicity relationships were found to be determined by the groups contained in that portion of the molecule added to the hexamethylenetetramine nucleus.

We can regard the material here presented merely as a beginning, but we feel that such a treatment of the problem as here presented may ultimately result in an accumulation of data which will be of value in the systematic search for substances which may be used in the control of experimental bacterial infections. Before passing judgment, however, on the chances offered by the further development of the quaternary salts of hexamethylenetetramine, the behavior of these substances in the animal organism should be studied in order to determine whether the bactericidogenic group in itself is sufficiently resistant towards metabolic changes. Otherwise these compounds as a class would be bactericidally inert *in vivo*.

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

II. THE RELATION BETWEEN CONSTITUTION AND BACTERICIDAL ACTION IN THE SUBSTITUTED BENZYLHEXAMETHYL-ENETETRAMINIUM SALTS.

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Hexamethylenetetramine, as a tertiary nitrogen compound, reacts readily with benzyl chloride, bromide, or iodide, and their numerous nucleus substituted derivatives to form quaternary salts. The results obtained in the study of the bactericidal properties of such substances are the subject of the present communication. In these compounds, the structure of which may be represented as follows,

$$CH_2 \diagdown \diagup Cl$$
$$(C_6H_{12}N_4)$$
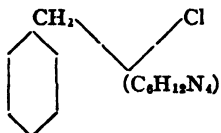
it is seen that by means of a CH_2 side-chain the hexamethylenetetramine molecule is linked to a benzene nucleus. By the use of a great variety of substituted benzyl halides it was found possible to prepare for study a variety of hexamethylenetetraminium salts[1] in which the benzene nucleus was varied at will in the character, number, and position of the different atoms and groups introduced. By this procedure the opportunity was afforded of studying the effect of chemical constitution upon bactericidal action in a uniform series of substances.

Because the number of substances involved in the investigations

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[1] For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 659; 1915, xxi, 465.

described in these papers was so large, it was found necessary for the practical execution of the bactericidal tests to adopt a scheme which would involve the least amount of work and still furnish a satisfactory measure of the activity of the substances. For this reason the drug dilutions, which as a rule started with 1: 200, were doubled in each successive dilution so that the series 1: 200, 1: 400, 1: 800, 1: 1,600, etc., were the concentrations with which the observations were made. With this scheme it is seen that as the dilutions increase the differences between them become greater, making it possible to regard the figures obtained only as rough approximations to the true values. In the strictest sense account should be taken of the molecular weights of the substances in a direct comparison of their bactericidal properties, but with the dilution scheme here employed this was deemed unnecessary.

In spite of the crudity of our figures it will be seen that certain relationships between the constitution and the bactericidal action are plainly in evidence. The results given below clearly demonstrate that by the addition of hexamethylenetetramine to benzyl chloride a bactericidal substance is obtained, and that by the substitution in the benzene nucleus of different atoms and groups this action may be altered at will, the resulting effect depending upon the number, character, and position of these substituents. In this class of compounds we possess a new group of bactericidal substances in which the hexamethylenetetramine nucleus is directly responsible for their bactericidal character.

<div align="center">EXPERIMENTAL PART.[2]</div>

Technique.—A strain of *Bacillus typhosus* which had been growing on artificial media for several years and which is a good agglutinator was used in testing the germicidal effects of the compounds.

0.5 or 1 per cent solutions of the substance to be tested were made up in physiological salt solution and filtered immediately through a Berkefeld N filter. With sterile salt solution the dilutions of 1: 200, 1: 400, 1: 800, 1: 1,600, 1: 3,200, 1: 6,400, and 1: 12,800 were made and

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[2] Some of the tests with *B. typhosus* were performed by Dr. Paul F. Clark, now of the University of Wisconsin, to whom we are greatly indebted for furnishing us with the results of his experiments.

all brought to a temperature of 37°C.   To 4 cc. of each dilution, there was added 0.5 cc. of a 24 hour broth culture of *Bacillus typhosus*, and the tubes were put into an incubator or water bath at 37°C. for 4 hours.   At the end of this time, one small loopful was taken from each tube and plated in plain agar.   It was found necessary to incubate the plates for 48 hours before counting, because some of the colonies did not grow out in 24 hours.   Control plates under the same conditions usually contained about 1,000 colonies.   Duplicates were run in each case.

Table I presents the bactericidal results obtained with the different preparations tested upon *Bacillus typhosus* by the technique described above.   As stated in the introduction, the number of preparations tested and the pressure of other work rendered impossible a detailed study of each substance employed in the tests, so that the figures here given can be regarded only as approximations to the true bactericidal powers of the substances in question.   In most instances the figures as given are, if anything, too low.   A consideration of the scheme of dilutions employed will show how great the underestimation of the true bactericidal power might be.   For example, in the case of the *o*-nitrobenzylhexamethylenetetraminium chloride, given in Table I, the greatest dilution in which this substance killed all the bacteria in 4 hours was 1: 3,200.   The next higher dilution tried was 1: 6,400 and this was found ineffective.   But if this compound could really kill in a dilution of 1: 5,000 or even 1: 6,000, the value obtained as a result of the scheme of dilution used would be only 1: 3,200.   For this reason we must regard the values given only as relative.   In spite of this, the alteration in character or position of the substituents in the nucleus was accompanied by changes in the bactericidal action which were too marked to be masked by the dilution scheme employed.

The tests with hexamethylenetetramine itself and the simple aliphatic quaternary salt methylhexamethylenetetraminium iodide showed them to be devoid of action in a dilution of 1: 200.   By the substitution in the latter compound of the methyl by the benzyl group the customary influence of the aromatic nucleus was observed. Although not a strong bactericide, the benzyl salt was found to kill all the bacteria present in a dilution of 1: 200.   This bactericidal

TABLE I.

Substance.	Killed B. *typhosus* in 4 hrs. at 37°C. in a dilution of 1
Hexamethylenetetramine...	+*
Methylhexamethylenetetraminium iodide.................................	+
Benzylhexamethylenetetraminium chloride................................	200
o-methylbenzylhexamethylenetetraminium chloride........................	3,200
m- " "	800
p- " "	800
3, 5-dimethylbenzylhexamethylenetetraminium chloride....................	400
o-chlorobenzylhexamethylenetetraminium "	1,600
p- " "	800
o-bromobenzylhexamethylenetetraminium "	1,600
p- " "	200
o-iodobenzylhexamethylenetetraminium bromide..........................	1,600
p- " "	1,600
o-cyanobenzylhexamethylenetetraminium chloride........................	3,200
p- " "	400
o-nitrobenzylhexamethylenetetraminium "	3,200
m- " "	400
p- " "	1,600
2, 4-dinitrobenzylhexamethylenetetraminium "	3,200
o-methoxybenzylhexamethylenetetraminium "	+
p- " "	200
2, 3-dimethoxybenzylhexamethylenetetraminium chloride..................	+
3, 4- " "	200
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride..............	200
5-nitro-2-methoxybenzylhexamethylenetetraminium "	400
3-nitro-4- " "	800
2-nitro-3, 4-dimethoxybenzylhexamethylenetetraminium "	3,200
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide...........	1,600
4-acetoxy-3, 5- " "	1,600
2-acetoxy-3, 5-dimethyl-4, 6-dibromobenzylhexamethylenetetraminium bromide..	1,600
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride..........	+
3-carboxy-4-oxybenzylhexamethylenetetraminium "	1,600
3-carbomethoxy-4-oxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carboxybenzylhexamethylenetetraminium "	400
2-methoxy-5-carbomethoxybenzylhexamethylenetetraminium "	400
o-acetaminobenzylhexamethylenetetraminium chloride....................	800
p- " "	+
1, 2-xylylenedihexamethylenetetraminium dichloride......................	12,800
1, 3- " "	6,400
Mesitylylenedihexamethylenetetraminium "	12,800

* + indicates growth after exposure to a dilution of 1:200.

power was further developed by the introduction into the nucleus of various atoms and groups, resulting in the series of substances given in Table I. A study of these brings out the following relationships.

The methyl, chlorine, bromine, iodine, cyano, and nitro groups were all found to increase the bactericidal power of the parent unsubstituted benzyl compound. This behavior of the alkyl, halogen, and nitro group has been frequently observed with other types of organic bactericides; for instance, in the case of the phenols. However, this effect may by no means be regarded as inevitable, as there are many bactericidal substances the power of which is in no way influenced by the introduction of these groups. Examples of this will be found among other types of hexamethylenetetraminium salts to be described in the following paper.

A perusal of the table will show the interesting influence of the position of the substituent upon the bactericidal action. In most cases the *ortho* substituents were found to be more active than either the *meta* or *para* compounds. The regularity of this rule, at least as far as the present series was extended, is striking. The *ortho* methyl, chloro, bromo, nitro, and cyanobenzyl salts were all more active than their other position-isomers.

With this type of hexamethylenetetraminium salt the methoxy group is, on the whole, without influence on the bactericidal effect, if indeed not detrimental. In those compounds which contain the nitro group besides the methoxy group the influence of the former appears to dominate. The 2-nitro-3,4-dimethoxybenzyl derivative was found to equal in effectiveness the 2-nitrobenzylhexamethylenetetraminium chloride. In the same way the 3-nitro-4-methoxy compound was bactericidally about as powerful as the 3-nitrobenzyl salt.

The optimum effect in varying substituents was apparently obtained with the mono- substituted benzyl compounds. In the few cases in which the dimethyl, dibromo, and dinitro derivatives were made, it was found that no advantage as regards the bactericidal value was to be gained, as a rule, by the multiplication of groups. At the same time the greater the number of substituents, particularly in the case of the nitro and halogen compounds, the less was the solubility in water. .

In one direction, however, at least when *Bacillus typhosus* was used

as the test object, it was found that a distinct advantage was to be gained by the multiplication of groups; namely, in those compounds in which hexamethylenetetramine was introduced twice into the side-chains. Such salts were obtained by the addition of two molecules of hexamethylenetetramine to ω_1,ω_2-dichloro-*o*-xylene, ω_1,ω_3-dichloro-*m*-xylene, and ω_1,ω_3-dichloromesitylene. These substances possess the following structural formula, in which, of course, the relative positions of the side-chains are different in each case.

$$CH_2\,(C_6H_{12}N_4)\,Cl$$
$$CH_2\,(C_6H_{12}N_4)\,Cl$$
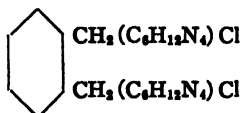
These compounds are the strongest bactericides of this group.

Here it should be emphasized that the bactericidal results obtained with this group of substances refer only to their behavior towards *Bacillus typhosus*. As the work developed, other species of bacteria were made the object of an occasional test, but owing to the incompleteness of the results obtained, and to the fact that the technique was varied, it has not been deemed advisable to enlarge on these results in the present paper. It may, however, be said that in general this class of substances was considerably less effective against the streptococcus and meningococcus, but that the results with the gonococcus approached those obtained with *Bacillus typhosus*. This particular group of hexamethylenetetraminium salts cannot, therefore, be regarded as general disinfectants. As a matter of fact, there are but few, if any, organic bactericides which act uniformly against all species or strains of bacteria. A few cases have been selected in Table II to afford a comparison of the effects of several of these salts upon different microorganisms. With the streptococcus, meningococcus, and gonococcus the technique was altered, the time of exposure of the bacteria to the drug being changed to 3 hours and the temperature to 20°. Such a change in technique should, of course, alter the results, but our experience has shown that this rarely exceeded the space of one whole dilution. It is seen from the table that the nature of the substance used determined the effect upon a particular microorganism. A constant relation between the resistances of the various types of organisms is out of the question. The far greater effectiveness

of the two dihexamethylenetetraminium salts against *Bacillus typhosus* is striking. These substances may be classed as "partially specific" for this species.

TABLE II.

Substance.	Killed *B. typhosus* at 37°C. in 4 hrs. in dilution of 1:	Killed streptococ- cus at 20°C. in 3 hrs. in dilution of 1:	Killed meningococ- cus at 20°C. in 3 hrs. in dilution of 1:	Killed gonococ- cus at 20°C. in 3 hrs. in dilution of 1:
Benzylhexamethylenetetraminium chloride............................	200	+	400	800
p-methylbenzylhexamethylenetetramin- ium chloride........................	800	+		
o-bromobenzylhexamethylenetetramin- ium chloride........................	1,600	+	+	1,600–3,200
o-cyanobenzylhexamethylenetetraminium chloride............................	3,200		400	1,600
p-cyanobenzylhexamethylenetetraminium chloride............................	400		400	800
o-methoxybenzylhexamethylenetetramin- ium chloride........................	+*	+	200	800
3, 4-methylenedioxybenzylhexamethyl- enetetraminium chloride.............	200	+	1,600	800
o-nitrobenzylhexamethylenetetraminium chloride............................	3,200	3,200	800	800
m-nitrobenzylhexamethylenetetraminium chloride............................	400		1,600	1,600
2-acetoxy-3, 5-dibromobenzylhexamethyl- enetetraminium bromide..............	1,600	3,200	800	800
2-acetoxy-3, 5-dimethylbenzylhexamethyl- enetetraminium chloride	+	1,600	800	800
m-xylylenedihexamethylenetetraminium dichloride..........................	6,400	200	+	1,600
Mesitylylenedihexamethylenetetraminium dichloride	12,800	+	400	400

* + indicates growth after exposure to a dilution of 1:200.

From the consideration of the above observations we feel justified in attributing essentially to the hexamethylenetetraminium group the property of determining the bactericidal character of this class of compounds. For direct comparison with another basic side-chain *p*-nitrobenzylpyridinium chloride was prepared. This was found to be ineffective towards *Bacillus typhosus* even in a concentration of 1: 200

after 4 hours' contact.   The corresponding hexamethylenetetramine quaternary salt killed *Bacillus typhosus* in a dilution of 1: 1,600 in 4 hours.

The function, however, of determining the extent and character of this bactericidal property must be attributed to the substituting groups and to the positions occupied by them in the benzene nucleus to which the hexamethylenetetramine is linked.   Our experience has shown that such groups likewise decide other biological properties of this class of substances.   Without stopping here to deal at length with the toxicity experiments it may be said that in general the toxicity of these compounds is determined by such groups.   For example, whereas the *o*-nitro benzyl derivative could be given to mice intravenously in amounts up to 500 mg. per kilo, the 2,3-dimethoxybenzyl derivative was found to be fatal in 0.1 of this dose.

SUMMARY.

By the addition of substituted benzyl halides to hexamethylenetetramine, a series of quaternary salts of this base was obtained. These salts represent a new group of organic bactericides.   The results obtained in the tests with these substances upon *Bacillus typhosus* have demonstrated the existence of direct relationships between chemical constitution and bactericidal action within the series.

The bactericidal character is directly attributable to the presence of the hexamethylenetetramine nucleus.   The degree of the bactericidal action, however, is determined by the position, character, and number of the groups substituted in the benzene nucleus.

By the introduction of the methyl, chlorine, bromine, iodine, cyano, and nitro groups into the benzene nucleus of the parent benzyl hexamethylenetetraminium salt, the bactericidal power of this compound was notably enhanced.   The substitution of these groups in the *ortho* position almost invariably resulted in substances which were more active than their *meta* or *para* isomers.   The introduction of the methoxy group was without marked effect.

Several substances in which two hexamethylenetetraminium sidechains occurred were found to be the most active of the substances of this series when tested against *Bacillus typhosus*.   Comparative tests with other bacterial types demonstrated that these compounds possessed a marked degree of specificity for *Bacillus typhosus*.

THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

III. THE RELATION BETWEEN CONSTITUTION AND BACTERICIDAL ACTION IN THE QUATERNARY SALTS OBTAINED FROM HALOGENACETYL COMPOUNDS.

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In the course of the biological investigations of the substituted benzyl quaternary salts of hexamethylenetetramine discussed in the previous paper, it was found that the further extension of this chemical type was no longer advisable, in spite of the fact that within this group substances had been found which possessed considerable bactericidal power against *Bacillus typhosus*. This was due partly to the inaccessibility or insolubility of the further preparations of this type which seemed theoretically indicated. Our attention was in consequence turned to the problem of finding a new scheme of chemical procedure which would furnish the possibility of new leads and of broader development. This was found in the readiness with which hexamethylenetetramine as a tertiary base reacted with halogenacetyl derivatives to form soluble quaternary salts with the following general structure,

$$R \cdot OC \cdot CH_2 \diagdown \diagup Cl \quad (C_6H_{12}N_4)$$
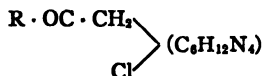
in which R might represent the radical of any aliphatic or aromatic primary or secondary amine R'NH, R'$_2$N, R'R''N, or of any alcohol or hydrocarbon. Because of the general nature of this reaction and the practically limitless number of chemical possibilities offered, it was found possible to furnish the most varied chemical groupings

with the hexamethylenetetramine molecule by the use of the halogen-acetyl side-chain.[1]

Without anticipating the detailed discussion of the observations to be found in the experimental part, the following remarks may be taken to sum up the principal results of the work presented in this communication.

As a result of experiments on a large number of drugs of this type, the general statement may be made that hexamethylenetetramine, when combined with halogenacetyl compounds in the form of quaternary salts, gives rise to a new group of organic bactericides. Hexamethylenetetramine may, therefore, be described as a definite bactericidogenic group. The extent of the bactericidal power of these substances is, in a measure, controlled by the general character of the molecule or of the particular groups contained therein. In addition, the employment of several species of microorganisms has served the purpose of bringing out many examples of partial specificity, at least one substance with a high degree of specificity being found for each of the four species of bacteria used.

The fact that this specificity shifted from one group of bacteria to another with the change in the chemical composition of the radical added to the hexamethylenetetramine, demonstrated that, in contradistinction to the bactericidogenic character, the specificity relations were determined, not by the hexamethylenetetramine nucleus, but by the added radicals.

From the facts set forth above it will be seen that the program presented in the introductory paper has been partially fulfilled. Thus it was found possible, by starting with the molecular grouping furnished by hexamethylenetetramine, to add the most varied atomic groupings with the aid of the $- CH_2CO -$ group, which served merely as a connecting link. The chemical differences in these added groups caused the wide variations observed in the bacteriological results.

In these observations, which must be regarded only as a beginning, we thus see that once in the possession of a biologically active or potentially active molecular group, it is possible, without produc-

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[1] For the chemistry of these substances and the reference to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, xx, 685; 1915, xxi, 103, 145, 403, 439, 455, 465.

ing profound chemical changes in the molecule itself, to equip it with a reactive side-chain which in turn will react with other molecular groups and which will furnish the necessary biological properties.

In the present paper we wish to present the results of the bactericidal tests performed with these preparations upon *Bacillus typhosus*, streptococcus, meningococcus, and gonococcus. Here, as in the previous communication, the large number of tests which were made necessitated the early adoption of a rough scheme of drug dilutions. For this reason what was said in the former paper regarding the accuracy of the results must be reiterated here. At best the figures given may be considered as rough approximations. In spite of this, however, in many instances pronounced evidence of the influence of constitution on bactericidal action appeared.

EXPERIMENTAL PART.[2]

Technique.—In testing the germicidal efficiency of the compounds to be described below, 0.5 or 1 per cent solutions of each substance, according to the solubility, were made in distilled water and filtered through a Berkefeld N filter. The other dilutions were prepared from this stock solution by the use of sterile distilled water as a diluent.

The series 1: 200, 1: 400, 1: 800, 1: 1,600, 1: 3,200, 1: 6,400, and 1: 12,800 was employed for the tests. In some cases the sparing solubility of the substance necessitated the omission of the lower dilutions.

5 cc. of each dilution were placed in wide mouthed tubes and the temperature was brought to 20°C. To each of these tubes the bacterial suspensions were added.

In the case of the *Bacillus typhosus* 0.1 cc. of a 24 hour broth culture was added to each of the tubes containing the dilutions of the compound. After 3 hours a standard 4 mm. loop of the mixture was plated in order to determine the number of living organisms. The plates were incubated for 48 hours before counting. Another loopful from the same tube was taken at practically the same time and inoculated into tubes containing 10 cc. of plain broth and the tubes were

incubated for 48 hours. It was found that at a certain dilution, using the plating method, there was a very abrupt falling off in the number of colonies in the plates. This point was always marked in the broth tubes by clear-cut differences in appearance of the incubated tubes. The organisms in the lower dilution produced marked turbidity and in the next higher dilution remained absolutely clear. It was apparent that from 50 to 80 organisms were necessary to inoculate a 10 cc. broth tube, so that anything below this number would not show in this culture media. On the other hand, each organism in the plates produced a colony. Having found at the beginning of the work that this point of abrupt decrease in the number of colonies, using the plate method, came always at the same dilution indicated by no growth in the tubes, the plate method was no longer used on account of the large number of drugs tested.

In the streptococcus tests one or two drops of a 24 hour bouillon culture of an ordinary hemolytic strain of streptococcus were added to each dilution tube. After 3 hours at 20°C. a loopful was taken from the bottom of each tube and plated in plain agar or blood agar. A bacterial control was run in distilled water and plated immediately after mixing and also at the end of the incubation period. By such a control it was possible to estimate the percentage of killing when complete killing did not occur. The plates were incubated at 37°C. for 18 hours and the results recorded.

In the case of the meningococcus the tests were made by Dr. Wollstein. Average 24 hour growths of the microorganism on sheep serum agar were washed off with 2 cc. of sterile distilled water. 0.5 cc. of this well mixed suspension was added to each tube containing the compound dilution. This was allowed to stand for 3 hours at 20°C. Then 0.2 cc. of each tube was planted on sheep serum agar slants. These were incubated for 48 hours and the readings taken. Controls accompanied each experiment.

For the gonococcus tests conducted by Dr. Pearce an adult strain of the organism was employed. 24 hour growths on ascitic veal agar were washed off with 3 to 5 cc. of·normal salt solution. The exact amount of salt solution depended upon the profuseness of the growth. The object was to obtain a decidedly cloudy but not milky suspension of the bacteria. 0.5 cc. of this suspension was then added to the tubes containing the substance dilutions and allowed to stand at

20°C. for 3 hours.   0.2 cc. was then pipetted from the bottom of the
tubes and planted on an ascitic veal agar slant and incubated for 48
hours.   The readings were then taken.   Controls were run in each
experiment.

In all the above experiments the tests were run in duplicate.

TABLE I.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	B. typhosus killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetamide..............	1,600	800–1,600	800	800–1,600
Chloroacetmethylamide........	1,600	800	3,200	1,600
Chloroacetethylamide.........	800	800–1,600	3,200	6,400
Chloroacetdimethylamide......	1,600	1,600	800	1,600
Chloroacetdiethylamide........	1,600	1,600	1,600	1,600
Chloroacetpiperidide..........	200	800		
β-iodopropionamide..........	+*	+	800	800

*+ indicates growth after exposure to a dilution of 1:200.

The results obtained with the hexamethylenetetramine quaternary
salts prepared from chloroacetamide and the chloroacetyl derivatives
of the simpler aliphatic amines will be found in Table I.   With but
few exceptions these substances were found to kill the organisms used
for the test in dilutions of at least 1: 1,600 in 3 hours at 20°C.   On the
whole, but little variation in action, at least of a magnitude which
could be detected by the dilution scheme employed, was obtained by
the addition of alkyl radicals to the amide nitrogen in the chloroace-
tamide salt.   The exceptions presented by the behavior of the methyl-
and ethylamide derivatives toward the meningococcus are worthy
of note.   With these substances the action was observed to be about
four times as great as that of the unsubstituted chloroacetamide salt
or its dimethyl derivative.   The unusual activity of the compound
obtained from chloroacetylethylamine against gonococcus is also
noteworthy.

When it is considered that substances of purely aliphatic nature
are represented in this series, the bactericidal power observed is quite
unusual.   Formaldehyde, which is considered one of the most pow-
erful of the aliphatic bactericides, when tested by the same tech-

nique was found to kill *Bacillus typhosus* in a maximum dilution of
1 : 1,200.   In addition, the molecular weights of these substances are
approximately ten times that of formaldehyde, so that if the com-
parison were strictly drawn the observed figures should be multi-
plied by ten.   On this basis they far exceed formaldehyde in molecu-
lar bactericidal power.   The activity of the substances of this group
as bactericides is attributable entirely to the presence in them of the
hexamethylenetetramine molecule.[3]

The comparative results obtained against *Bacillus typhosus* by the
same technique with other aromatic substances which have been re-
garded in the past as strong organic antiseptics are given in Table
II.   Unfortunately the tests were restricted to *Bacillus typhosus*.
Among the substances given in Table I and in those to follow, many
will be found which are as active or more active than any given in
this table.

TABLE II.

3 Hours at 20° C.

Substance.	B. typhosus killed in dilution of 1:
Formaldehyde...	1,200
Phenol..	+*
Lysol...	400
Trichlorophenol...	800
Tribromo-*p*-cresol...	1,600
Tetrabromo-*o*-cresol.......................................	1,600
Tribromo-*m*-xylenol..	1,600
Gentian violet..	3,200

*+ indicates growth after exposure to a dilution of 1:200.

In an attempt to determine what influence might result from the
substitution of the halogenacetic acid radical by that of other halo-
gen fatty acids, it was found that the higher α-halogen fatty acid

[3] There is evidence that the hexamethylenetetramine molecule does not always
persist as such when its quaternary salts are dissolved in water, but undergoes a
decomposition which yields methylene derivatives of the corresponding amines.
The relation of these substances to the observed effects we shall make the subject
of a subsequent communication.

derivatives failed to react with hexamethylenetetramine. The derivatives of β-iodopropionic acid, however, were found to react smoothly with the base to give quaternary salts. The ineffectiveness of the salt obtained from β-iodopropionamide in particular served to indicate the scant promise offered by the further employment of this acid and that the best results would be obtained by the continued use of the halogenacetyl group.

In the logical development of the above substances of purely aliphatic origin, the effect of the introduction of the aromatic nucleus into the alkyl group situated on the amide nitrogen was studied. The opportunity for this was furnished by the quaternary salts obtained from the chloroacetylbenzylamines possessing the following structural formula:
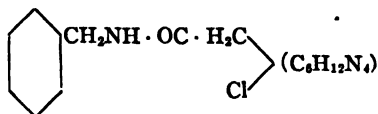
It was thought that here the usual antiseptic influence of the aromatic nucleus would appear, but, as will be seen from the results presented in Table III, this did not conform to the expectations. In interpreting the results, however, the greater molecular weights of these substances should not be neglected. Nevertheless, in those cases in which the observations fell below 1:800 the chemical structure alone must be held responsible. Owing to the irregular character of the fluctuations observed it is difficult to deduce from this table any general relationships between the chemical constitution and the bactericidal power. In the case of the streptococcus and gonococcus, however, the introduction of the methyl group seemed to enhance the activity. The methoxy derivatives also appeared to be more effective than the corresponding acetoxy compounds. It is possible that a series of salts prepared from the mono- substituted benzylamines would have afforded a less confusing and more comparable group of substances for study. The difficulty of procuring such material and the pressure of other work were obstacles to the further extension of this chemical type.

On turning to the more easily accessible chloroacetylanilines, a series of substances was obtained which afforded ample opportunity·

TABLE III.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	B. typhosus killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylbenzylamine......	1,600*	800	1,600	800
Chloroacetyl-o-methylbenzyl-amine...................	1,600*	3,200	1,600	3,200–6,400
p-acetaminoiodoacetylbenzyl-amine...................	800*	200	1,600	800
1-methyl-4-acetaminochloro-acetylbenzylamine.........	+†	800	800	1,600
1, 2-diacetoxychloroacetyl-benzylamine..............	800*	800–1,600	800	400
1, 2-dimethoxychloroacetyl-benzylamine..............	1,600*	800	400	800
1-acetamino-4-ethoxychloro-acetylbenzylamine.........	800–1,600	800–1,600	400	800
β-acetoxy-α-naphthochloroacet-ylbenzylamine............	800	1,600	800	800
β-methoxy-α-naphthochloro-acetylbenzylamine.........	1,600	3,200	1,600	1,600
m-carbethoxychloroacetylben-zylamine.................	800	400	400	800
m-carbamidochloroacetylben-zylamine.................	+		3,200	1,600
Diethylaminoethyl ester of m-carboxychloroacetylbenzyl-amine...................	200	800		

* Tests in these cases were made at 37° C.

† + indicates growth after exposure to a dilution of 1:200.

for ascertaining the influence of the introduction of groups into the benzene nucleus. These substances possessed the following structural formula, in which any group X may occur in the *ortho*, *meta*, or *para* positions:

$$NH \cdot CO \cdot CH_2 \diagup Cl \quad (C_6H_{12}N_4)$$
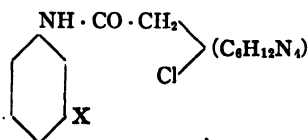
Table IV shows that the linkage of hexamethylenetetramine by means of the chloroacetyl radical with the simpler aromatic amines

TABLE IV.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaniline.................	800	1,600	1,600	3,200
Chloroacetyl-*o*-toluidine............	1,600*	1,600	1,600	1,600
Chloroacetyl-*p*- "	800	800	1,600	3,200
Chloroacetyl-*m*-4-xylidine...........	800	1,600	1,600	3,200
Chloroacetyl-*ψ*-cumidine............	800	1,600	3,200	3,200
Chloroacetyl-*α*-naphthylamine........	800	800	800	1,600–3,200
Chloroacetyl-*β*- "	1,600*	800	6,400	6,400
6-chloroacetylaminoquinoline	3,200	3,200	1,600	1,600
Chloroacetyl-*o*-chloroaniline.........	1,600	1,600	800	3,200
Chloroacetyl-*p*-bromoaniline.........	1,600	1,600	1,600	3,200
Chloroacetyl-5-iodo-*o*-toluidine.......	1,600	800	1,600	3,200
Chloroacetyl-*m*-nitraniline...........	3,200	3,200	3,200	3,200
Chloroacetyl-*m*-nitro-*p*-toluidine......	800			
o-chloroacetylaminophenol	1,600	800	800	3,200–6,400
Chloroacetyl-*o*-anisidine............	800		3,200	1,600
Chloroacetyl-*p*- "	3,200	1,600		
β-iodopropionyl-*o*- "	+†	200	400	400
Chloroacetylmethylaniline		1,600	800	1,600
Chloroacetyldiphenylamine..........	400	200	1,600	1,600
p-chloroacetylaminobenzoic ethyl ester	1,600	3,200	1,600	3,200
Chloroacetylnovocaine..............			1,600	3,200–6,400
o-chloroacetylaminobenzyl alcohol	800	800–1,600	800	800
o- " benzoate...	800	3,200	1,600	
o-chloroacetylaminophenyl " ...	200	1,600	800	3,200
o- " *p*-nitro- benzoate........................	800	1,600–3,200	1,600	1,600
m-chloroacetylaminoacetophenone....		1,600–3,200	800	1,600

* Tests were made at 37° C.

†+ indicates growth after exposure to a dilution of 1:200.

produced a group of substances possessing definite bactericidal properties. Contrary to the results obtained with the substituted benzyl compounds described in the previous communication, it was found that alterations in the benzene nucleus by the usual substituents did not result in sharp differences in the bactericidal effect, at least of a magnitude which could be revealed by the scheme of dilutions employed. Many instances are to be found, however, in which the ac-

tivity of the simplest member, the salt obtained from chloroacetyl-aniline, has been definitely improved. Among these may be mentioned the substances obtained by the introduction of the methyl, chlorine, bromine, iodine, and nitro groups. Such chemical variations, however, did not always result in an improvement. In many cases the bacteria were killed in dilutions of 1: 3,200 or even 1: 6,400. On the whole, of the microorganisms employed, the gonococcus was the least resistant towards the members of this group. In the absence of indications of a more decided character or of greater regularity there was little assurance of obtaining more powerful bactericides by the further use of these substituents.

The results, however, assumed a different character by the adoption of a new type of variation within this group of substances. This consisted in the use of the dialkylamino group as a substituent in the nucleus of the parent chloroacetylaniline quaternary salt. These substances were prepared by the reaction of the chloroacetylamino-dialkyl anilines with hexamethylenetetramine and possessed the following structure:

$$NH \cdot CO \cdot CH_2 \diagdown \diagup (C_6H_{12}N_4)$$
$$Cl$$
$$N \diagup R \diagdown R$$
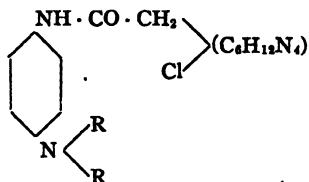
in which R may be methyl, ethyl, etc. The bactericidal results are given in Table V.
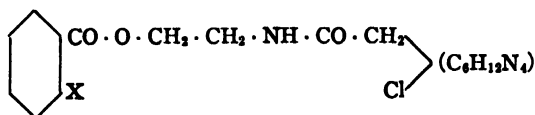
TABLE V.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	B. typhosus killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningo-coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylaminodimethylaniline	+†	800–1,600	1,600	1,600
p-chloroacetylaminodiethylaniline.		3,200–6,400	1,600	3,200
p-chloroacetylaminodipropylaniline*		6,400	3,200	6,400
p-chloroacetylaminobenzylethylaniline* . .		6,400	6,400	12,800
m-chloroacetylaminodimethylaniline		1,600	400	400

* In these cases, owing to the sparing solubility in water, one mol. of N HCl was used to dissolve the substances.

† + indicates growth after exposure to a dilution of 1:200.

Our attention was first called to the desirability of developing this series by the apparent partial specificity of the dimethyl compound for the streptococcus as compared with the effect observed upon *Bacillus typhosus*.   Later its effectiveness against the meningococcus and gonococcus was found to be equally marked.   The later members of the group were obtained by replacing the methyl groups by ethyl, propyl, and benzyl.   In these tests *Bacillus typhosus* was unfortunately omitted.   We are therefore in no position to state whether this organism is more resistant to these substances as a class.

The regularity of the response to this particular chemical alteration is strikingly shown by these results.   A progressive improvement occurred in the bactericidal action upon all three species of bacteria upon proceeding from the dimethyl to the diethyl, dipropyl, and finally the benzylethyl derivatives, and this in spite of the constant increase in molecular weight.   If the increase with each dilution had not been so great, it is probable that all the columns of the table would have shown the regularity of the gonococcus results.   The striking feature of the observations is the magnitude of the effect produced by such slight alterations in a complicated molecule.   The inferior results obtained with the *meta*-dimethylamino compound, the last in the table, would indicate that the relative positions occupied by the substituents in the nucleus are modifying factors.   ·

The efficacy of these groups was still further demonstrated by their use in another class of substances obtained by the addition of hexamethylenetetramine to chloroacetylaminoazobenzene derivatives, in which the base was attached by the chloroacetylamino side-chain to one nucleus and the dialkylamino group to the other as presented in the following formula:
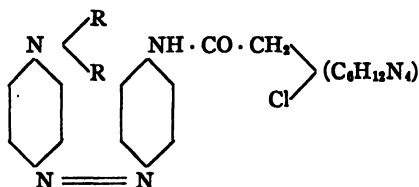
In Table VI the salt obtained from chloroacetylaminoazotoluene, which contains no dialkylamino group, is first given as an

TABLE VI.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	B. *typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylaminoazotoluene.............	3,200	3,200	3,200	1,600
p-chloroacetylaminobenzeneazo-*p'*-dimethyl-aniline*...............................	800	12,800	3,200	3,200
p-chloroacetylaminobenzeneazo-*p'*-diethyl-aniline..................................	+‡	12,800	1,600	800
p-chloroacetylaminobenzeneazo-*p'*-dipropyl-aniline*...............................		12,800	800	800
p-chloroacetylaminobenzeneazo-*p'*-benzyl-ethylaniline*...........................		12,800	1,600	800
Benzeneazo-*m*-chloroacetylaminophenol†.....		3,200		

* In these cases 1 mol. of N HCl was employed to dissolve the substance.
† Solution made by the use of 1 mol. N NaOH.
‡+ indicates growth after exposure to a dilution of 1:200.

object for comparison.[4]   The action of this substance upon the different species of bacteria was fairly uniform.   The introduction, however, of the dimethylamino group into that position in the molecule farthest removed from the location of the hexamethylenetetramine nucleus resulted in a marked difference.   But slight alteration, if any, was observed in the meningococcus, a slight improvement towards the gonococcus, and a considerable reduction in the action upon *Bacillus typhosus*.   With the streptococcus, however, the change was profound.   The action was increased at least fourfold.   The efficacy of this type of chemical modification against the streptococcus was still further confirmed by the replacement of the dimethyl group by the diethyl, dipropyl, and benzylethyl groups.   These variations produced compounds which, in spite of the increased molecular weight, exhibited a degree of action of the same order.   On the other hand, when tested against the other organisms they were found to be bactericidally less active than the dimethyl compound.   We have here an interesting instance of specificity for streptococcus.

[4] The simpler chloroacetylaminoazobenzene derivative could not be conveniently used because of its sparing solubility in water.

Here again the hexamethylenetetramine molecule was found to be a factor in bringing out this effect. This was directly demonstrated in the following manner: p-aminobenzeneazodiethylaniline may be considered as the third substance mentioned in the table deprived of hexamethylenetetramine and the $-CH_2CO-$ radical which serves only as a connecting link. This dye was found to kill the streptococcus in a maximum dilution of 1: 3,200, an effect which, though marked, is still but one-fourth of the result obtained with its hexamethylenetetraminium salt.

TABLE VII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	B. *typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
p-chloroacetylamino-p',p''-tetramethyl-diaminotriphenylmethane (p-chloro-acetylaminoleukomalachite green)*	400	12,800	200	200
o-chloroacetylamino-p',p''-tetraethyldi-aminotriphenylmethane*..............		12,800	6,400	1,600
p-chloroacetylamino-p',p''-tetraethyldi-aminotriphenylmethane*..............		51,200	1,600	1,600
Chloroacetyltriphenylamine.............		3,200–6,400	800	800

* In these cases 1 mol. of N HCl was employed to dissolve the substance.

The group of substances given in Table VII headed by the hexamethylenetetramine quaternary salt of chloroacetyl-p-aminoleukomalachite green,
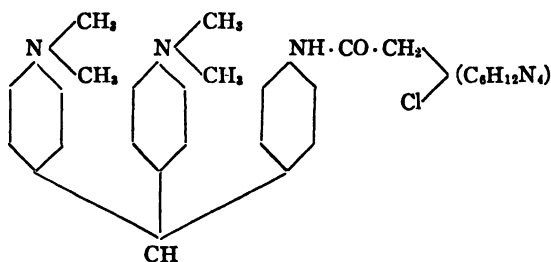
afforded the opportunity of still further testing the value of dialkylamino compounds against the streptococcus. This salt, as well as its homologs, displayed a marked specificity for this microorganism, kill-

ing it in a dilution of at least 1: 12,800, whereas the effect on other the forms was relatively weak.   In the next two compounds the methyl groups were changed to ethyl groups, and in one case the chloroacetyl-amino radical was shifted to the *ortho* position.   The dilution of 1: 12,800 was usually the highest dilution employed for the tests in the routine procedure, but fortunately the experiments performed with the third substance were carried further.   This preparation was found to kill the streptococcus even in a dilution of 1: 51,200, making it probable that the first and second substances also would have been found to kill above 1: 12,800.

That here also the hexamethylenetetramine molecule is an essential factor was proven as follows.   The first substance given in the table when deprived of this base and its connecting group is nothing else than *p*-aminoleukomalachite green.   This substance required a concentration of at least 1: 800 to kill the streptococcus in 3 hours. In other words, the hexamethylenetetraminium salt derived from it was at least sixteen times more active.

In the course of the present work our interest was centered for a time in the study of the biological properties of the hexamethylene-tetramine quaternary salts obtained from the chloroacetylalcamines. Our attention was attracted first to this group of substances by the powerful bactericidal properties of the simplest representative, that obtained by the addition of hexamethylenetetramine to chloroacetyl-aminomethanol.[5]

$$\text{HO} \cdot \text{CH}_2 \cdot \text{NH} \cdot \text{CO} \cdot \text{CH}_2 \diagdown \!\!\!\!\!\diagup\!\!\!\!\! \text{(C}_6\text{H}_{12}\text{N}_4\text{)}$$
$$\text{Cl}$$
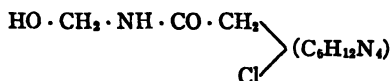
This substance, which possesses the above structural formula, is the first given in Table VIII.   It is seen to possess a marked action against all the species used with the exception of the streptococcus. Because of the unusual effectiveness of this product it was hoped that its suitable chemical variation might lead to a series of very active substances.

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[5] This substance was first prepared by Einhorn and Göttler (*Ann. d. Chem.*, 1908, ccclxi, 150), who also recognized its antiseptic properties.
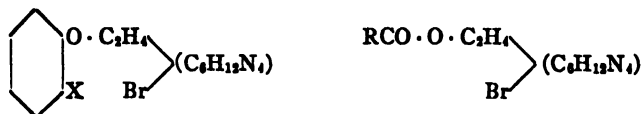
TABLE VIII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1·	Gonococcus killed in dilution of 1:
Chloroacetylaminomethanol................	3,200	400–800	6,400	6,400
Iodoacetylaminoethanol...................		400	1,600	800
Chloroacetylaminoisopropanol.............		200		
δ-chloroacetylamino-*n*-butanol.............		400		
β-chloroacetylamino-γ- "		800		
γ-chloroacetylamino-β-methyl-β-butanol....		400		
α-phenyl-α-oxy-β-chloroacetylaminoethane...		1,600		1,600
β-phenyl-β-oxy-α-chloroacetylaminopropane..		800		
Chloroacetylaminoethyl ether.............		1,600		
Chloroacetyl-*o*-methylphenoxyethylamine ...		800		

The chemical development of this substance was attempted in two ways: first, by the replacement of its methanol group by the ethanol, propanol, butanol, etc., radicals; and second, by the acylation of the methanol hydroxyl group with various acid radicals. In the latter scheme, however, chemical difficulties were encountered which compelled the use of its homologs, in particular the ethanol derivative, as the basis for the study of the effect of acylation.

Table VIII presents the behavior principally towards streptococcus of the substances obtained by the first method of chemical variation. The results show that the first member of the group, the salt obtained from chloroacetylaminomethanol, is the most powerful, so that no bactericidal increase was to be gained by such a chemical procedure.

Quite a different result was obtained by the use of the second scheme of chemical variation, as will be seen in Table IX. Unfortunately the inaccessibility of the acylated methanol derivatives made impossible a direct comparison of the effect of acylation upon the chloroacetylaminomethanol salt itself. The results must therefore be referred to the parent unacylated alcamine compound in question for a strict comparison. The structural formula of this group of substances may be represented as follows, X being any substituting group:

$$\text{(benzene ring)} \begin{matrix} CO \cdot O \cdot CH_2 \cdot CH_2 \cdot NH \cdot CO \cdot CH_2 \\ X \end{matrix} \Big\rangle_{Cl} (C_6H_{12}N_4)$$

A glance at the table will show that we have here another group of hexamethylenetetraminium salts with strong bactericidal properties surpassing in this respect the parent chloroacetylalcamine compound.

TABLE IX.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaminoethyl benzoate............	800	400	1,600–3,200
" o-methylbenzoate....	1,600	3,200	1,600
" p- " ...	3,200	1,600	800
" β-naphthoate.......	3,200	6,400	6,400
" o-nitrobenzoate.....	800	3,200	1,600
" p- "	6,400	6,400	3,200
" p-methoxybenzoate..	3,200		
" acetylsalicylate......	800		
" p-diethylaminoben-zeneazo-p'-carboxylate	6,400–12,800	400	400
Chloroacetylamino-γ-propyl p-nitrobenzoate .	1,600		
" -γ- " p-methoxyben-zoate.................................	1,600–3,200	800	1,600–3,200
Chloroacetylethylaminoethyl p-nitrobenzoate	3,200.		
Chloroacetylphenylaminoethyl p- "	800		3,200–6,400

By the introduction of the simplest aromatic acid, benzoic acid, the bactericidal power of the parent iodoacetylaminoethanol salt was doubled, except for the meningococcus. The use of the substituted benzoic acids, such as the methyl, nitro, and methoxybenzoic, and naphthoic acids, in most cases still further improved the action. In the case of the nitrobenzoates the *para* compound seemed more effective than its *ortho* isomer. With the methylbenzoyl derivatives the *para* compound was also more active towards the streptococcus than its *ortho* isomer. With meningococcus and gonococcus the reverse was the case. The specificity of the *p*-diethylaminobenzeneazo-*p'*-carboxylate for streptococcus was to be expected from the results already discussed in connection with Table VI. This substance possesses the

diethylamino group. With the few acids studied, the best results were obtained with the *p*-nitrobenzoyl and *β*-naphthoyl compounds. The results yielded by the use of other alcamines, such as aminopropanol, ethylaminoethanol, etc., would seem to indicate that the optimum effect is to be obtained with the aminoethanol series.

In this group of substances but a few representatives were made and tested. By the use of numerous other acids a much broader series might be developed for study with the possibility of obtaining more active preparations. However, the observations obtained with this small group of substances serve to demonstrate again to what extent the bactericidal effect may be altered by relatively small changes in the molecule. Here, as in the case of the benzylhexamethylenetetraminium salts discussed in the previous communication, the degree of action is determined by the character and position of the substituents in the benzene nucleus. The main source of the bactericidal effect, however, is still the hexamethylenetetramine molecule.

TABLE X.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylurea	3,200	800–1,600	1,600	1,600
α-chloroacetyl-β-methylurea.............	800	400–800	1,600	1,600
α-chloroacetyl-β-benzylurea	400	400	800	1,600
Chloroacetylurethane...................	400		1,600	800

Still another type of hexamethylenetetramine quaternary salt included in the investigations was that represented by the compound obtained by the reaction of chloroacetylurea with hexamethylenetetramine. In Table X it is seen that for a purely aliphatic substance it exhibited a strong bactericidal power. It was hoped that by turning to the substituted ureas this action might be improved. The experience with the methyl and benzyl compounds, however, showed only a diminution of the activity.

Up to this point the substances which have been the subject of discussion were all quaternary salts obtained from halogenacetylamino compounds. Two other types of substances were included in the

TABLE XI.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	B. typhosus killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetone......................	1,600*			
ω-bromoacetophenone...............	1,600*	3,200	1,600	1,600
p-methyl-ω-bromoacetophenone......	1,600	800	800	1,600
p-ethyl-ω- "	800			
1, 2-dimethyl-ω- "	3,200	3,200	3,200	6,400
1, 3-dimethyl-ω- "	1,600		800	1,600
m-nitro-ω- "		1,600		
p-methoxy-ω- "	800	800	3,200	6,400
p-ethoxy-ω- "	800	1,600	1,600	1,600–3,200
p-acetamino-ω- "	800	3,200	1,600	3,200
3-acetamino-4-methyl-ω- "			800	800
3-acetamino-4-tolyl ω-iodoethyl ketone	800	1,600–3,200	12,800	12,800
1, 2-diacetoxy-ω-iodoacetophenone....	400	1,600	800	800
β-[ω-bromoaceto]-quinaldine.	200	3,200	3,200	3,200

* Tests were made at 37° C.

study in which hexamethylenetetramine was joined by means of the halogenacetyl group first to hydrocarbons and then to alcohols. The first of these groups, which was prepared by the addition of halogen ketones to hexamethylenetetramine, may be represented by the following formula:
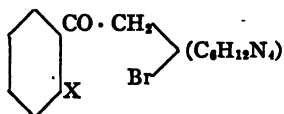
The results of the experiments made with these substances are contained in Table XI. The bactericidogenic property of hexamethylenetetramine was again demonstrated. The first member, the salt obtained from chloroacetone, was found to kill *Bacillus typhosus* in a dilution of 1:1,600, which is again striking for an aliphatic substance. Among the aromatic representatives the majority killed one or another of the species tested in dilutions of 1:1,600 or more. The behavior of the 1,2-dimethyl-ω-bromoacetophenone and p-methoxy-ω-bromoacetophenone derivatives toward the gonococcus and the action of the salt obtained from 3-acetamino-4-tolyl ω-iodoethyl

ketone on the gonococcus and the meningococcus are worthy of note. It is seen that the chemical constitution of the compounds determines in a degree the bactericidal power, but any definite regularity is far from apparent. As in many instances to be seen in the other tables, the result of a particular chemical variation upon the bactericidal power varies according to the organism used for the test. An interesting instance of the influence of the relative positions occupied by substituents in the benzene nucleus upon the bactericidal effect is shown by the differing action of the 1,2- and the 1,3-dimethyl-ω-bromoacetophenone salts towards the meningococcus and the gonococcus. The former substance is four times more active than the latter.

The results yielded by the salts obtained from halogenacetyl esters are given in Table XII. The ease of saponification of this chemical

TABLE XII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	B. *typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Ethyl bromoacetate..................	400	1,600	800	800
Phenyl "	800	3,200	1,600	3,200
Bornyl "	3,200			
Menthyl "	800	1,600–3,200	1,600	1,600
Ethyl β-iodopropionate................	+*	200	200	400
Anisoylglycol chloroacetate.............	1,600	1,600	1,600	1,600
p-nitrobenzoylaminoisopropyl chloroace- tate................................	1,600	1,600		1,600

*+ indicates growth after exposure to a dilution of 1:200.

type limited its more extended development. The table demonstrates the bactericidogenic properties of hexamethylenetetramine in this combination also. The relatively low bactericidal power of the β-iodopropionyl derivative is also in line with the results obtained with other derivatives of this acid.

In the course of the work still other connecting groups than the halogenacetyl radical were used in order to combine hexamethylenetetramine in the form of quaternary salts with other molecular group-

ings. Bromoethyl alcohol by virtue of its alcoholic hydroxyl group may combine with acids to form bromoethyl esters or may be considered the mother-substance of the bromoethyl ethers. These bromoethyl derivatives react readily with hexamethylenetetramine, giving the two following classes of salts:

The results obtained with the first of these, the bromoethyl ether salts, are given in Table XIII. It is to be observed that this type was, on the whole, most active against the meningococcus and the

TABLE XIII.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Phenyl bromoethyl ether............	200	+	400	800
o-methylphenyl bromoethyl ether	+*	400	1,600	1,600–3,200
m- " " "	400		1,600	1,600
p- " " "	400		400	800
α-naphthyl bromoethyl ether.........	+	800	3,200	12,800
β- " " "	800	1,600–3,200	1,600	3,200–6,400
p-bromophenyl " "	200	·+	3,200	1,600
Tribromo-*p*-cresyl " "		· 3,200	800	800
o-acetaminophenyl " "			400	400
p- " " "			200	200

gonococcus. The partial specificity of the α- and β-naphthol bromoethyl ether salts for the gonococcus is especially noteworthy. The α-compound, which killed the gonococcus in a dilution of 1:12,800, was ineffective against *Bacillus typhosus* in a concentration of 1:200. These instances, together with the other substances mentioned in the table which were found to kill one or the other microorganism in dilutions of 1:1,600 or 1:3,200, still further indicate how general in character is the bactericidogenic property of the hexamethylenetetramine molecule

TABLE XIV.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	*B. typhosus* killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Bromoethyl acetate......................	+*		800	400
" benzoate.....................	200	400	400	800
" *p*-nitrobenzoate...............	+		800	800
Bromoethylphthalimide	+	+	400	800

* + indicates growth after exposure to a dilution of 1:200.

In the case of the bromoethyl ester salts (Table XIV) the introduction of the hexamethylenetetramine molecule is seen to be considerably less effective. However, here again the best results were obtained with the meningococcus and the gonococcus. The last substance in the table is not an ester but a bromoethylamino compound. This also was most active against the meningococcus and gonococcus. It would seem from both this series and the previous group of substances that there is something in the chemical nature of the salts obtained from bromoethyl compounds which renders them most active against these two species of bacteria. Although not as marked, this may be considered analogous to the specific effect of the dialkylamino group upon the streptococcus.

It is highly probable that the further development of any of the leads which have been indicated in these papers might eventually furnish more active preparations which would be of chemotherapeutic value.

. In conclusion we wish to present the results obtained in a few experiments on the effect of serum and protein on the bactericidal action of several of the compounds mentioned in the preceding tables. It so happened that in these serum-compatibility tests the technique employed was that described in the preceding paper. For this reason the parallel observations made with solutions of the compounds in physiological salt solution are a dilution higher than those to be found in the preceding tables. Table XV presents the results of these tests.

TABLE XV.

Hexamethylenetetramine quaternary salt of	B. typhosus killed in 4 hrs. at 37° in a dilution of 1:	
	In physiological salt solution.	In horse serum.
Chloroacetyl-o-toluidine................................	3,200	3,200
Chloroacetylaminomethanol............................	6,400	3,200
ω-bromoacetophenone.................................	1,600	800
1, 2-diacetoxychloroacetylbenzylamine...................	1,600	800

It is seen that in the case of the salt obtained from chloroacetyl-o-toluidine the action was not inhibited by serum.   In the other cases the observed effect was reduced by half in the presence of serum.   It is possible that in these cases the apparent inhibition was accentuated by the dilution scheme employed, and that in reality but little relative inhibition occurred.   With a few other compounds of this class tested by a different technique a much greater relative inhibition of the bactericidal action was observed.   From these experiments we may at any rate conclude that the bactericidogenic hexamethylenetetramine portion of the molecule does not in itself cause serum-incompatibility.   The source of this must be sought in the remainder of the molecule.

TABLE XVI.

Hexamethylenetetramine quaternary salt of	Gonococcus killed in 2 hrs. at 20° in a dilution of 1:	
	In aqueous solution.	In 5 per cent sodium caseinate solution.
Chloroacetyl-β-naphthylamine..........................	3,200	1,600
p-methoxy-ω-bromoacetophenone........................	6,400	6,400
Chloroacetylnovocaine................................	6,400	6,400
α-naphthyl bromoethyl ether...........................	12,800	6,400
Choloroacetylaminoethyl-p-nitrobenzoate................	3,200	3,200
3-acetamino-4-tolyl ω-iodoethyl ketone..................	12,800	1,600

In Table XVI will be found the results of a series of tests in which the substances were dissolved in a 5 per cent sodium caseinate solution. The gonococcus was here used and the technique employed was the same as that described in the other gonococcus tests.   In only one

case, the last given in the table, was any marked inhibition to be observed. With the remaining substances mentioned relatively little or no inhibition was observed.

SUMMARY.

The extension of the study of the quaternary salts of hexamethylenetetramine to those obtained by the addition of this base to the most varied types of substances containing aliphatically bound halogen has demonstrated that the introduction of the hexamethylenetetramine nucleus in this manner results in the production of bactericidal substances or enhances the bactericidal action if already present.

In particular it was found possible by the use of the halogenacetyl group, XCH_2CO, as a connecting link, to furnish primary and secondary aliphatic and aromatic amines, alcohols, and hydrocarbons of the most varied character with the hexamethylenetetramine molecule and to study the relation between chemical constitution and bactericidal action in the series of substances so prepared. Because of the variety of chemical types studied, the results are too involved for a detailed summary here.

Many of the substances were found to be very powerful bactericides, and in a number of instances derivatives of purely aliphatic nature were found to possess an unusual bactericidal power.

Bacillus typhosus, streptococcus, meningococcus, and gonococcus were the microorganisms used for the tests, and striking instances of partial specificity were observed. This specificity was found to favor not one species alone, but instances were found in which each of the types of bacilli was shown to be especially susceptible to one or another of the particular types of compound employed. The source of this partial specificity is to be sought not in the hexamethylenetetramine nucleus itself but in the molecule to which it is attached.

The action of some of the substances was tested in the presence of serum or protein and was found to be not at all or only slightly inhibited. In other cases marked inhibition occurred. The factors controlling the serum- or protein-compatibility of these substances are likewise to be sought in that portion of the molecule other than the hexamethylenetetramine.

ARE FUNCTION AND FUNCTIONAL STIMULUS FACTORS IN PRODUCING AND PRESERVING MORPHO-LOGICAL STRUCTURE?

BY EDUARD UHLENHUTH, PH.D.

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Since the days of Lamarck the attempt has often been made to explain the genesis of the morphological structure of organisms through the theory of adaptation. A special form of this theory is that of "functional adaptation" which was formulated under this name by Wilhelm Roux about 1880, and was later elaborated by that investigator in an extremely extensive and thorough manner.

The most striking organic structures are those which like the bones seem to be constructed on a definitely purposeful plan, offering the largest amount of strength with the smallest amount of material. Other organs, such as the muscles, increase in size as a result of increased function. Roux named this phenomenon "functional adaptation," while the structures underlying this principle he described as "functional structures." He made a number of exceedingly careful anatomical studies of such "functional structures." Endeavoring to explain the genesis of such seemingly purposeful structures from a purely mechanical standpoint, he found that they possessed exactly that construction which was to be expected from a mathematical calculation based on the principle of functional adaptation.

In order to make clear the development of functional adaptation, Roux fell back upon the most primitive particles of living matter. In his opinion some of these particles have been adapted to respond to functional stimuli, that is, they show a greater tendency to proliferate in the presence of functional stimuli than in their absence. Thus, those elements which were subjected to stimuli soon predominated over those which were not thus exposed.

If functional structures consisted of such particles, they would show the following characteristics: first, enlargement (functional

hypertrophy) with increased stimulus; second, atrophy from in-
activity upon cessation of the stimulus; third, regeneration of organs
of functional structure to the condition of the original structure
would only be possible in the presence of function and functional
stimuli (functional regeneration); and fourth, the successful trans-
plantation of functional structures would only be conceivable if
after transplantation the organs involved were supplied with an
appropriate amount of functional stimulus. The principles, there-
fore, necessary for an acceptance of the theory of functional adapta-
tion are: functional hypertrophy, atrophy from inactivity, func-
tional regeneration, and functional transplantation. (Various other
factors which are similarly instrumental in this connection will be
dwelt upon more extensively in a subsequent paper.) Should it now
appear that these essential principles in so-called functional structures
are non-existent, the theory of adaptation would then fail to ade-
quately account for so-called functional structures.

Before giving the results of my experiments I wish to say a few
words about the general value of Roux's theory of functional
adaptation.

This theory at a cursory glance has many merits, and at the time
when first introduced by Roux it marked a substantial advance,
inasmuch as it showed that it was possible to produce highly pur-
poseful structures through the influence of purely mechanical princi-
ples. On this basis the theory has become a material factor in con-
nection with the investigations and studies of many workers in the
realms of pathology and physiology.

Roux's theory, however, has great disadvantages, one of which is
its extremely complicated and extensive terminology. It is probably
owing to the extreme obscurity of the doctrine that comparatively
few investigators, apart from the originator of the theory, have famil-
iarized themselves with it and fully understood its principles. As a
result of this practically the only valuable work along this line has
been carried on under the direct control of Roux himself, while in
contrast with the comparatively scanty publications emanating
from Roux's laboratory an amazingly voluminous mass of literature
has been supplied by outside writers, all of whom were under the
impression that they were contributing something to Roux's theory,

but who in reality had hardly grasped more than a few but imperfectly comprehended terms connected with the essential problem. These for the most part misleading publications have caused more error than progress in experimental work. I shall endeavor to prove this point in a subsequent and more extensive article.

A second disadvantage of Roux's theory is the fact that the extent of the field in which it is applicable becomes more and more restricted with the increasing number of "experimental" investigations bearing directly upon this problem. Consequently, in course of time the phenomena which are not in harmony with this principle of functional adaptation increase in number, although they may be explained, together with other phenomena, from another point of view.

However, by far the weightiest objection to the theory is that it threatens to become more and more of a stumbling block to workers who are setting out to investigate the problems of organic and inorganic life from a common viewpoint. Nowadays theories such as that of function and functional stimulus can hardly be reconciled with a chemico-physical view of the life processes; for the whole underlying principle of the theory of adaptation does not lend itself to methods of measurement. For this reason a detailed revision of the theory of functional adaptation has become necessary and will be published in a later communication.

In the present paper I shall report on the results of a few experiments which I began five years ago with the above mentioned object in view. As they are at the present moment sufficiently advanced to allow of a survey of the whole point at issue and to show that they are qualified to throw some light on the problem of functional adaptation, a preliminary discussion of these experiments may be warranted.

The experiments in question were performed on the transplanted eye of *Salamandra maculosa*. The eye of a larva was transplanted into the neck of another larva, where after a few days partial or complete disappearance of the retina resulted, ending finally in complete degeneration. *The remarkable fact was that this degeneration was followed later by a complete regeneration of the transplanted eye, even in the dark.*

My experiments were conducted with the following considerations

in view. The retina is one of those structures which according to Roux's definition should be called a functional structure. But in accepting this definition one need not necessarily assume that these structures are the result of functional stimulus, for such structures may have been predetermined in the embryonic stage. They develop independently of functional stimulus until they reach the third period—the so-called functional period.[1] Functional structures can only exist during this period if they are supplied with a sufficient amount of functional stimulus; otherwise they undergo atrophy through inactivity. From Roux's point of view it would appear probable that the morphogenesis of the eye of *Urodela* is only partially determined by inheritance, and in accordance with this determination it would reach the same stage of development as that attained by *Proteus anguineus*. The further development of the eye, comprising the formation of the rods and cones, that is the true functional parts, would be brought about by the penetration of the rays of light through the skin of the salamander, to its ovaries, and would therefore be the outcome of functional stimulus. Secerov[2] asserts that the skin of *Salamandra maculosa* permits of the penetration of approximately 1/173 of the quantity of light in which the animal lives.

The eyes of *Proteus*, which inhabits dark caves, must therefore remain in the primitive *Proteus* stage, according to the statement of Kammerer,[3] who believes he has demonstrated that further differentiation can only occur if *Proteus* be kept in the light. Thus, he ascribes the process of full differentiation of the eye in the case of several *Proteus* which were kept in the light, directly to the influence of function, and Roux is apparently of the same opinion. This point will be discussed by the writer in a later paper.

[1] See, for example, Roux, "Die Entwicklungsmechanik, ein neuer Zweig der biologischen Wissenschaft," Vorträge und Aufs. über Entwickl.-Mech. d. Organismen. 1905, Heft I., p. 94, note 11. Also Roux, "Die vier Hauptperioden der Ontogenese, sowie das doppelte Bestimmtsein der organischen Gestaltungen," *Mitteilung der naturforschenden Gesellschaft*, Halle a.d.S., 1911, I., p. 1.

[2] Secerov, S., "Die Umwelt des Keimplasmas. II. Der Lichtgenus im Salamandra-Körper," *Arch. f. Entwcklungsm.*, 1912, XXXIII., 682.

[3] Kammerer, P., "Experimente über Fortpflanzung, Farbe, Augen und Körperreduktion bei *Proteus anguineus* Laur. Zgl. Vererbung erzwungener Farbveränderungen." III. Mitteil., *Arch. f. Entwcklungsm.*, 1912, XXXIII., 349.

My own observations on the transplanted eye of *Salamandra* soon convinced me that this case lends itself very well for the test of the theory of functional adaptation.

First of all I severed the optic nerve, a procedure which according to general opinion should induce permanent degeneration of the retina as a result of the eye becoming isolated from the brain. In all previous operations of this nature, where, however, the eye remained in its normal position, a reunion of the amputated stumps of the nerve took place, so that it was natural to suppose that the re-connection of the eye with the brain brought about regeneration of the retina. In my own experiments I obviated the possibility of such subsequent reunion of the eye with the brain by transplanting the eye to an abnormal position (in the neck of the salamander). But regeneration took place in spite of this fact. The point of chief interest, however, is the fact that by means of this operative measure, which, as has been demonstrated in 95 per cent. of the cases, excludes reunion of the eye with the central nervous system, the eye is permanently deprived of functional power.

It is thus obvious that in these eyes no function was possible and the experiment therefore shows that a whole series of phenomena, hitherto designated as cases of functional adaptation, requires a different explanation. We will now discuss these phenomena in greater detail.

I. In about a week after transplantation of the eye into the neck of the salamander the retina had degenerated to such an extent that in many cases only the peripheral part of the retina, which was not differentiated in layers, had survived.[1] But in spite of its permanent isolation from the brain and despite the fact that the eye was permanently deprived of function, the retina from this time on began to show signs of regeneration and the transplanted eye began to receive a progressively improved supply of blood, so that in a comparatively short time (from 4 to 6 weeks) it had regained a perfectly normal structure.

II. This regeneration of the transplanted eye even takes place when the organ is deprived of functional stimulus by light. A series

[1] E. Uhlenhuth, "Die Transplantation des Amphibienauges," *Arch. f. Entwcklungsm.*, 1912, XXXIII.

of salamanders operated on in the above-described manner, were placed in a dark room where neither red nor white light could penetrate to their eyes; but in spite of this fact the transplanted eyes regenerated and developed a normal retina.

These experiments show that the "quality" of this process, namely, regeneration as such, is independent of any sort of functional influence. We are here dealing with a case of simple regeneration, such as is found in many organs, not with *functional* regeneration, such as we might expect to find in so-called functional structures. Of course this fact does not warrant us in entirely rejecting the theory of functional adaptation, for the possibility must not be ignored that although regeneration occurs in eyes treated in this way as a result of the agency of certain other factors, nevertheless degeneration brought about by atrophy through inactivity might follow later, as a result of the permanent lack of function and functional stimulus, a possibility which would be expected to arise according to Roux's theory.

III. But secondary degeneration as a result of atrophy from inactivity failed to occur in my experiments, even when the eyes were permanently deprived of function, as occurred in the "light" series.

IV. Degeneration similarly failed to occur in the transplanted eyes which were permanently deprived of both function and functional stimulus, namely in the "dark" series. These eyes, although severed from the brain and in permanent darkness, grew and metamorphosed simultaneously with the normal eyes of the hosts.[1]

Up to the present time I have had at my disposal preparations of eyes of the "dark" series which were preserved 15 1/2 months after transplantation; at that time the hosts were about 21 months old, and all the structures, with the exception of the sex organs, were perfectly developed. The retinas of these transplanted eyes were found to be normal in every detail.

In addition to the above I have a preparation of an eye of the "light" series, which was preserved 3 1/4 years after transplantation, at which time the sex organs were also fully developed. Accord-

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[1] E. Uhlenhuth, "Die synchrone Metamorphose transplantierter Salamanderaugen (Zugleich, Die Transplantation des Amphibienauges. II. Mitteil.) *Arch. f. Entwcklungsm.*, April, 1913, XXXVI., 211.

ing to Roux the eye of an amphibian should by that time already have entered the functional period, as is believed to have been proved in the case of the eye of the *Proteus*. Nevertheless, the old transplanted eyes were also found to be normal, and the functional parts of the retina, viz., the rods and cones, were present and well developed.

The above results, namely, the permanent preservation in a normal condition of transplanted eyes, prove beyond any doubt that the so-called functional structures of the eye do not undergo atrophy through inactivity, even if they are kept under extremely unfavorable conditions and are deprived of all function and functional stimulus.

This fact alone is sufficient to show that atrophy from inactivity, which is one of the fundamental postulates of Roux's theory, is by no means a phenomenon of general occurrence which takes place in all so-called functional structures permanently deprived of functional stimulus, as was supposed to be the case.

As far as regeneration is concerned, the experiments mentioned above only serve to show that regeneration in itself is independent of function and functional stimulus.

V. I am, moreover, able to demonstrate that the "quantity" of the regenerative process in the eyes, viz., the rapidity of this regeneration is not influenced by functional stimulus, viz., light.

A certain number of animals (260 in all) of both the light and the dark series were preserved at certain intervals of time and the transplanted eyes were cut in sections. Eyes preserved at equal intervals of time were then compared with each other. It was found that the transplanted eyes of the same series which had lived on the host for the same period of time may show considerable differences in the rapidity with which they undergo regeneration, even if they are subjected to equal conditions as far as functional stimulus is concerned. These differences must therefore be caused by other non-specific factors. In order to ascertain how far-reaching is the influence of light, it was necessary to determine the average rapidity of regeneration in every group of eyes of the same series and make a curve for each series. Although these curves are not yet completed, the results thus far obtained show no differences between light and dark series at all. Even the quantity of the regeneration is therefore uninfluenced by light.

From the above data we must draw the following conclusions: *Functional adaptation plays no part either in transplantation or in regeneration of the retina; nor is it a factor which determines either the quality or the quantity of these processes.*

This, of course, does not mean that regeneration or transplantation of the eye cannot be influenced at all by chemical or physical factors. On the contrary, as is shown by the differences between transplanted eyes of the same series, examined at equal intervals of time after transplantation, the quantity of regenerative processes, viz., the rapidity of regeneration is subject to variation by one or more factors. The point of importance is the fact that these factors are not connected with the specific functional stimulus, viz., light. Apparently they are the same factors which also affect the rapidity of the regenerative process of other organs. The factor concerned is probably the length of time since circulation in the transplanted eye was reëstablished.

Hitherto the regeneration of the retina has been considered as being different from the regeneration of the bones. It was supposed that a bone could only regenerate its architectural structure if in use, otherwise the result would not be a normal bone but a disorderly bony mass.

Aug. Bier,[1] however, has shown that even the bones do not regenerate as a mere indefinite mass of bone if kept without functional stimulus, but that on the contrary, in the absence of any such stimulus, they resume their original functional structure to the minutest details. In certain experiments a considerable part of a human tibia was removed. Although these bones were not exposed to any function, the tibiæ after a certain length of time assumed their normal shape and structure, but only if they were supplied with nourishment in a proper way, and if sufficient space was left for them to regenerate the missing part.

The same is true of the tendons. H. Triepel[2] showed that the tendo Achillis of a cat can regenerate only tendon tissue, irrespective of the presence or absence of functional stimuli.

[1] Aug. Bier, "Beobachtungen über Knochenregeneration," *Arch. f. klin. Chir.*, Dec., 1912, c, 91.

[2] Triepel, H., "Selbstständige Neubildung einer Achillessehne," *Arch. f. Entwcklungsm.*, Aug., 1913, XXXVII., 278.

Our own experiments now prove the same principle also in the case of the eye of *Salamandra maculosa;* this organ regenerates its functional structures in the absence of functional stimulus, and furthermore it retains its structure permanently, despite the permanent absence of functional stimulus. For a long time it was believed that a bone only regenerated a structureless mass of callus in the absence of function, and according to this theory it would be assumed that an eye if once degenerated would in the absence of light regenerate undifferentiated retina cells, such as we find in the normal *Proteus* eye. Nevertheless, both eye and bone regenerate the normal and fully differentiated structure, even in the absence of functional stimulus.

We have seen that in my experiments the velocity of the process of regeneration was not influenced by function; and even if this had been the case it could not be used as a proof in favor of functional adaptation. There are a number of well known morphogenetic processes, the rapidity of which can be accelerated by light, although light bears no relation to the function of the developing organ, that is to say, is not a functional stimulus. The most striking experiments made in this connection are those of J. Loeb,[1] in which he showed that the regeneration of the hydrants of *Eudendrium* is impossible in the absence of light. Nevertheless, we cannot call this a case of functional adaptation, because here light is obviously not a functional stimulus. On the other hand, development of the eyes of fish embryos cannot be prevented by the exclusion of the functional stimulus. But it is incorrect to ascribe this fact to the assumption that heredity may have fixed this character so far that development of an eye now occurs in the absence of light. For Loeb[2] has shown that it is very easy to prevent the development of these eyes by a number of different means, such as lack of oxygen, which is a non-specific, non-functional factor. Moreover, it is not necessary to point out that the influence of light on a photographic plate has never been considered to be a case of functional adaptation, although the sensitiveness of the plate to light is just as much re-

[1] Loeb, J., "Über den Einfluss des Lichtes auf die Organbildung bei Tieren," *Pflügers Arch.*, April, 1896, LXIII., 273.

[2] Loeb, J., "Heredity in Heterogeneous Hybrids," *Jour. of Morphol.*, March, 1912, XXIII., 1.

sponse to a physical factor as is the regeneration of *Eudendrium* or as might be the variation in the rapidity of eye-regeneration which although not found in our experiments, might possibly have occurred.

The theory of functional adaptation complicates instead of simplifying the problem. What we should emphasize is not the fact that the result of the response to light is different in the case of *Eudendrium* from what it is in the case of the regenerated eye or of the photographic plate, but the fact that these three phenomena all possess a common basis. It is obvious that all three are governed by the same laws, with which we are familiar from our knowledge of physics and chemistry; but these laws are free from such terms as function, functional stimulus, or any other stimulus, or the principle of adaptation. In order, therefore, to obtain a fertile method for attacking the problems confronting us we must constantly bear in mind the fact that the same laws expressed in the same terms can explain both organic and inorganic phenomena.

A CONTRIBUTION TO THE BIOLOGY OF PERIPHERAL NERVES IN TRANSPLANTATION.

II. LIFE OF PERIPHERAL NERVES OF MAMMALS IN PLASMA.

BY RAGNVALD INGEBRIGTSEN, M.D.

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PLATES 34 TO 41.

Nageotte[1] has demonstrated that in pieces of peripheral nerves removed from the living animal and kept *in vitro* under certain conditions, the first stages of Wallerian degeneration may occur exactly as in the peripheral part of a divided nerve in the living organism. The phenomena are identical, but the process takes place more quickly *in vitro*. The conditions required for this degeneration *in vitro* are the presence of a solution of sodium chloride with some salt of bivalent metals (calcium, strontium, or magnesium) in suitable proportions, such as Ringer solution.

Nageotte found that the incubation of nerve fibers in isotonic solution of sodium chloride for twenty-four hours left them unaltered, and if they were put in Locke's solution after several days they degenerated exactly as if they were put directly into this fluid. I have observed a typical Wallerian degeneration in nerve fibers incubated in homologous and heterologous serum at body temperature for twenty-four hours, and in this case, as well as in Locke's solution, the degeneration takes place much more quickly than in the organism. One would expect, therefore, a Wallerian degeneration as a result of the incubation of peripheral nerves in plasma, but this did not take place.

[1] Nageotte, J., *Compt. rend. Acad. d. sc.*, 1910, cl, 731; *Compt. rend. Soc. de biol.*, 1910, lxix, 556.

The experiments were performed in the following way. From a living rabbit pieces ½ to ⅔ cm. long were removed from the sciatic nerve, and the nerve fibers were dissociated in Ringer solution by means of needles. The fibers were put immediately into a drop of plasma on a cover-glass, which was inverted over a hollow slide, sealed with paraffin, and incubated at 38° C. The preparations were observed from day to day under the microscope on a heated table and no change in the fibers was noticed up to the 10th or 12th day after incubation (Figs. 1 and 2). In hardened and stained preparations the nerve fibers had the same appearance on the 6th, 7th, and 8th days as on the 1st day.

I attempted to produce a degeneration in the nerves by adding calcium chloride to the plasma, but the results were the same. One drop of a 1 per cent solution of calcium chloride was added to 5, 7, and 8 drops of plasma, and from each of these mixtures a series of preparations were incubated at 38° C. As a control 1 drop of 1 per cent solution of calcium chloride was added to 5, 7, and 8 drops of serum and to 5, 7, and 8 drops of Ringer solution in which nerve fibers were incubated.

In the nerve fibers from the calcium plasma no degeneration occurred, whereas the nerves from the calcium serum and calcium Ringer solution tubes developed a degeneration in the usual way.

It is well known that pieces of connective tissue, glandular organs, skin, etc., when incubated for some time in plasma give rise to a growth of tissue around the original fragment, in some cases of the same specific cells as those constituting the original piece, but more often built of cells resembling the connective tissue cell type. Accordingly, it was to be expected that in the experiments a growth of tissue would occur originating from the cells of Schwann, but this did not take place. I prepared 80 cultures of nerve fibers removed from the sciatic nerve of living rabbits and in none was growth observed up to the 16th day of incubation.

I then cut the sciatic nerve of a rabbit, closed the wound, and twenty-four hours later removed a piece from the peripheral part. From this piece of nerve which had developed the first faint changes

of the Wallerian degeneration 10 cultures were prepared.   No growth was observed from these nerve fibers.

Another series of 32 cultures was prepared, in the usual way with a hanging drop of plasma in hollow slides, from the peripheral part of a divided nerve 2 days after section.   There were no changes in the fibers, and no growth was observed up to the 16th day.

Twelve cultures were prepared from the peripheral part of a divided nerve 4 days after section, and there was no growth.

In a series of 36 cultures prepared from the peripheral part of a divided nerve 5 days after section different results were recorded. In 6 of these 36 cultures I observed a growth of characteristic appearance.

After incubating the cultures for 3 and 4 days a number of thin filaments consisting of light-breaking, slightly granular protoplasm were seen growing out from the cut ends of the nerve fibers.   In the beginning they have a conic or tapering shape ending in a point in which are seen ameboid movements, by means of which they emerge into the plasma, gain in length, and soon take on a more or less regularly cylindrical appearance measuring in width the same as or slightly less than the individual nerve fibers from which they have grown out. They branch freely, and from the 5th day they assume other characteristics, which are, however, best studied after fixation and staining.

Some of the cultures were hardened in formalin (2 per cent) and subsequently stained in hematoxylin.   Others were hardened by means of the Held pyridin method and stained by Cajal's silver nitrate method.

Preparations Stained by Hematoxylin.—(Figs. 3, 4, and 5.)   The original piece of nerve is 3 to 8 mm. long and about ½ to 1 mm. wide. In its two ends the individual nerve fibers are easily discernible and display the ordinary appearance of Wallerian degeneration from this stage (that is, on the 5th day).   Included in the sheaths of Schwann the following structures are seen: myelin ovoids containing fragments of the axis cylinder, and between 2 ovoids the nuclei of Schwann dislodged into the center of the fiber and embedded in protoplasm. From the cut ends of the individual nerve fibers the newly grown structures enter the plasma.   In many of the fibers that give rise to growth, the cross-section of the fiber made by its removal from the

animal has passed close by a nucleus of Schwann, and such a cell marks the end of the old fiber and the beginning of the young one. In only a few of the old fibers a nucleus of Schwann is not present at the end, but in these cases it is found in the fiber only a few microns further in toward the center.

When they reach the plasma, the newly grown protoplasmic structures which form a direct outgrowth from the perinuclear protoplasm (syncytium) of Schwann of the old fibers are slightly fibrillary or granular. They have a tapering shape and a width averaging at their starting point 6 to 8 microns; that is, slightly less than the fibers from which they have grown out. Their tree-like branches are much thinner than the main stem and end in a point or a little bulb, round or oval in shape. In some of the newly grown filaments from distance to distance a well outlined and darkly stained nucleus is seen. These nuclei are oval or fusiform with their longest diameter in the direction of the filament; their shortest (transverse) diameter is ordinarily larger than the filament itself and the presence of the nuclei in the filaments produces varicosities of a corresponding size.

In some of the filaments 3 to 4 nuclei appear in a row one after another embedded in a granular column of protoplasm without any membrane, the whole picture resembling the contents of the sheaths of Schwann in a degenerating nerve 14 to 15 days after section. To complete this likeness we find included in some of the grown out threads small agglomerations of fatty (myelin) globules of different volume from that of minute granules up to or even surpassing the size of a nucleus of Schwann, embedded in varicose accumulations of protoplasm and generally beside a nucleus of Schwann.

In some preparations the filaments after reaching the plasma anastomose between each other, and thus form a real framework-like syncytium (Fig. 4). After 6 days of incubation the newly grown filaments are about 500 microns in length.

Silver-Impregnated Preparations.—The Held pyridin method was found to be the only practicable one for fixing the cultures. It causes no precipitation in the plasma and the subsequent staining by Cajal's silver nitrate method has given good results.

The original fragments of nerves stain dark brown and the sur-rounding plasma a light yellowish brown. The filaments which have

grown out are slightly darker brown than the plasma and are easily discernible against this background. In no case did the newly grown filaments assume the black, or dark brown color which is characteristic of axis cylinders treated according to this method, which I have demonstrated growing out from pieces of cerebellum and spinal ganglia incubated in plasma.

Morphologically the preparations display the same appearance as the hematoxylin preparations.

The newly grown filaments emerge from the transversely cut ends of the nerve fibers, at the end of which a nucleus of Schwann surrounded by protoplasm is often observed; or such a cell is seen a little further in toward the center of the piece, or in the young fiber a few microns from its base. The filaments are of different sizes, measuring in width from 2 to 3 microns up to 8 to 9 microns at the point where they enter the plasma. From this point they steadily decrease in size and in their course through the plasma they curve and bend in various directions, although as a rule the main direction in the beginning of their growth at least is parallel to the nerve fibers of the original fragment. The filaments are for the most part unevenly shaped and provided with large and small varicosities. The large varicosities include one and sometimes two oval nuclei of the Schwann nucleus type, the small varicosities are formed by accumulation of granular protoplasm. Some of the largest filaments divide into two or three branches which part from the main stem at acute angles, usually leaving a little varicosity of protoplasm at the point of division. The finest branches are provided with minute varicosities and they end in a point or in a little granular protoplasmic bulb.

In a series of 20 cultures prepared from the peripheral part of a divided nerve 6 days after section a growth of filaments was observed in 5. The newly grown structures had the appearance described above (Figs. 6 and 7).

I prepared cultures from the peripheral part of a divided nerve, 8, 10, 13, 15, and 19 days after section. The results of these experiments are shown in Table I.

The following conclusions may be drawn from these experiments. Plasma cultures prepared from normal peripheral nerves give rise

to no proliferation of cells. Plasma cultures prepared from the peripheral part of a divided nerve within the first 4 days after section give rise to no growth. 16 per cent of plasma cultures prepared from the peripheral part of a divided nerve 5 days after section give rise to a proliferation in the plasma of the syncytium of Schwann, and in series of cultures prepared from such a divided nerve in Wallerian degeneration there is a direct ratio between the stage of the Wallerian degeneration counted in days and the percentage of positive cultures as far as the growth of syncytium of Schwann is concerned.

The growth in plasma of the syncytium of Schwann from nerve fibers in a Wallerian degeneration of a more progressed stage, from the

TABLE I.

Cultures of the Peripheral Part of a Divided Nerve in Different Stages of Wallerian Degeneration.

Stage of Wallerian degeneration (No. of days elapsed after section).	Total No. of cultures.	No. of cultures in which growth had occurred.	Percentage of growth.
days			
1, 2, and 4..............	54	None	0
5..............	36	6	16.6
6..............	20	5	25.0
8..............	20	6	30.0
10..............	20	8	40.0
13..............	24	. 11	45.8
15..............	21	- 16	76.0
19..............	17	14	82.3

13th day for instance, displays minor variations from what has been described concerning the growth from fibers in an earlier stage. The growth is more abundant and the rate of growth is quicker. The fibers are mostly parallel; they are much thinner in their whole length and are far more richly provided with nuclei of the ordinary oval Schwann nucleus type. Around many of these nuclei there is an agglomeration of fatty (myelin) granules embedded in scant protoplasm. The filaments from the later stages of Wallerian degeneration are not so liable to branch, and anastomoses are not seen (Figs. 8, 9, 10, and 11). In Cajal preparations from this stage too, the fibers stain light brown and none of them take the black or dark brown

color which is characteristic of the axis cylinders. Besides the filaments a migration and growth of ordinary connective tissue cells (Fig. 3) are found in many of the cultures.

I should mention here the results of my experiments on the cultivation of pieces of the central nervous system and spinal ganglia of young mammals. In 1913[2] I observed that the brains of chick embryos, of cats 6 weeks old, of rabbits 2 months old, and of dogs 3 weeks old, when cultivated in plasma developed long (1.25 mm.) protoplasmic filaments, which were studied mainly from living preparations and were interpreted as nerve fibers. Such structures developed also from spinal ganglia of rabbits 7 months old and from the spinal cord of cats 6 weeks old. In a later communication,[3] after having succeeded in staining the preparations by means of Cajal silver impregnation, I stated in confirmation of my preliminary report, that nerve fibers grow out from pieces of cerebellum and spinal ganglia of young cats and guinea pigs when cultivated in plasma, and that these nerve fibers did not anastomose, and extended into the plasma unaccompanied by structures of any kind.

Certain other phenomena of growth were also observed by means of the silver impregnation which made possible a discrimination between two different kind of filaments, one of which was nerve fibers and the other probably neuroglia. The proof that nerve fibers grew into the plasma was based upon the direct and continual extension of the black silver-stained nerve fibers of the original pieces into the plasma, where they formed evenly cylindrical or uneven and varicose, mostly branching filaments, which were never granular and did not anastomose.

Besides these fibers an abundant growth of syncytial protoplasm was observed. In unstained living preparations it is difficult if not impossible to tell whether a filament is a nerve fiber or a neuroglia fiber, the difference between them being rather insignificant aside from the anastomoses, which, judging from the stained specimens, frequently occur between the neuroglia fibers but never between the nerve fibers. In silver-stained specimens, however, there is a marked

[2] Ingebrigtsen, R., *Jour. Exper. Med.*, 1913, xvii, 182.

[3] Ingebrigtsen, R., *Jour. Exper. Med.*, 1913, xviii, 412.

difference between the grown out nerve fibers and the neuroglia syncytium.

As shown in Fig. 12, the filaments supposed to be neuroglia are not quite cylindrical but are unevenly shaped; they are provided with varicosities, and between the latter the protoplasm is granular. The main point, however, is the presence of anastomoses between different fibers, resulting in a real framework-like syncytium in the plasma. The difficulties encountered in the fixation of the cultures have made my experiments aiming to a coloration by means of a method supposed to be more or less specific for neuroglia entirely unsuccessful, so aside from the silver-stained preparations I have studied them by means of basic hematoxylin. I cannot, therefore, prove that the fibers just described are neuroglia, but indirect evidence supporting this view can be produced by excluding what we know they are not. Previous investigations (Carrel and Burrows, Ingebrigtsen) have shown that connective tissue and endothelial tissue grow in plasma in a manner quite different from the structures under consideration. As demonstrated from the silver preparations the nerve fibers growing out from ganglia cells also display a different aspect. And then the only conceivable idea concerning their origin is the assumption that these fibers are growing neuroglia tissue.

Fig. 13 A is produced by a combination of three photographs of a culture 3 days old from a piece of cerebellum taken from a young cat. The photographs have been focused on three different planes, following the fibers in their course from their beginning in a big cell of the cortex of cerebellum out to their branches and ends in the plasma. Fig. 13 B is a drawing reproduced from the combined photographs, bringing out details of the preparation which have been omitted in the photographs. These illustrations show how the protoplasmic filaments growing out of the big cell branch, anastomose between themselves, and with other fibers from the neighborhood form a real syncytium built of protoplasmic filaments of different size.

DISCUSSION.

The Wallerian degeneration of a peripheral nerve means the death and disintegration of the axis cylinders and myelin sheath.

From the work of Ranvier[4] we know that the process is influenced in its rate by various agencies; thus it occurs more quickly in young animals than in older ones, in vigorous and healthy individuals than in the sick, more quickly also in warm-blooded than in cold-blooded animals, and in mammals it occurs more quickly in rabbits, for instance, than in dogs.

But in any case the various stages of degeneration follow each other in a typical way quite different from that of a nerve in the dead body.

Mönckeberg and Bethe[5] have observed that the first stages of the Wallerian degeneration may occur in a dead animal when it is kept at body temperature for some hours, but that it stops within the first twenty-four hours. I have confirmed the results of these investigators and found that there is no progress of the degeneration of the nerves after twelve hours in a dead body kept at 38°. As the death of the individual is different from cellular death, the latter occurring later than the former, Mönckeberg and Bethe concluded that the Wallerian degeneration is a process connected with living tissue.

Merzbacher[6] confirming this view added that the occurrence of a Wallerian degeneration in a nerve means that this nerve is in a condition of survival.

Nageotte[1] succeeded in producing the first stages of Wallerian degeneration in nerves kept in survival *in vitro*, and his work has demonstrated that the segmentation of the axis cylinders and myelin sheaths is independent of, and does not, as believed by Ranvier, result from the functions of the protoplasm of the cells of Schwann. The conception of Nageotte concerning the segmentation of the myelin-clad axis cylinders is that the myelin sheath, which is built of living protoplasm rich in mitochondria, digests the axis cylinders in the closed ovoid cavities formed by its segmentation, dying later after having performed this function. This conception of the part played by the myelin sheath in the destruction of the axis cylinder has been adopted by Cajal. The autodigestion of the myelin-clad

[4] Ranvier, L. A., Leçons sur l'histologie du système nerveux, Paris, 1878.
[5] Mönckeberg, G., and Bethe, A., *Arch. f. mikr. Anat.*, 1899, liv, 135.
[6] Merzbacher, *Neurol. Centralbl.*, 1905, xxiv, 150.

axis cylinders develops *in vitro* in a solution containing sodium chloride and salts of some bivalent metal in certain proportions. This observation of Nageotte demonstrates that the segmentation of the myelin sheath obeys the laws of Loeb as does the life of living animals (Nageotte). It obeys also the other laws of biology. It does not occur in nerves kept at 0° nor in those kept at 45° C.

I have found that the segmentation of the myelin sheath is completely abolished in plasma. This does not mean that the nerves kept in plasma are dead; for we know (Carrel and Burrows[7] and Ingebrigtsen[8]) that plasma is by far the best medium for the maintenance of life of tissue outside of the body, and we have a direct proof of the survival of nerves kept in plasma from the fact that in this medium the cells of Schwann give rise to a rich proliferation. And nerve fibers incubated in plasma for twenty-four and forty-eight hours develop a Wallerian degeneration when incubated afterwards in Ringer solution. The proper ratio of calcium and sodium salts is present in plasma as well as in serum, and the addition of calcium chloride did not produce segmentation.

It is difficult to understand how Wallerian degeneration is inhibited in plasma. The reasonable conclusion seems to be that the nerve does not die and degenerate in plasma, because it exists there in extremely favorable conditions, more favorable than those prevailing in the organism, where the peripheral part of a divided nerve must degenerate.

Proliferation of the cells of Schwann does not take place, however, even in plasma unless the first stages of the myelin segmentation have developed in the fibers already before their implantation in the new medium. While fibers removed from the peripheral part of a divided nerve within the first 4 days after section give rise to no growth in plasma, a growth occurred in 16 per cent of the cultures prepared from nerve fibers on the 5th day of Wallerian degeneration, and from later stages an increasing percentage of positive cultures was recorded.

We know that in the peripheral part of a divided nerve in rabbits a hypertrophy of the protoplasm of the cells of Schwann is visible on

[7] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiv, 244.

[8] Ingebrigtsen, R., *Jour. Exper. Med.*, 1912, xvi, 421.

the 4th day; their multiplication begins about the 6th day, when the segmentation of the myelin sheath is well started. From these facts it may be concluded that the cells of Schwann are quiescent and unable to multiply up to a certain stage of myelin and axis cylinder segmentation, and that the latter and not the section of the nerve is the direct cause of their proliferation.

Our interpretation of the protoplasmic structures growing into the plasma from the cut ends of the nerve fibers seems to be conclusive; it is clear from the illustrations that they are direct outgrowths from the cells of Schwann, characterized by a tendency to form a syncytial framework. Is this an indication that such an outgrowth occurs from the cut end of a degenerating nerve (central end of peripheral segment) in the organism, and plays some part in the processes of union and regeneration? I have never seen it and I am not aware that it has been noticed by other observers. But it is probable that such a growth from the cells of Schwann of the peripheral part of a divided nerve extends into the scar tissue forming part of it. That structures of this kind have not yet been detected in the organism does not prove their non-existence. They stain faintly, and curving and bending through the scar tissue it may be difficult to bring out their true form.

The centrifugal orientation of the young axis cylinders growing out from the central part of a divided nerve has been explained by Forssman,[9] and his explanation has been accepted and modified by Cajal[10] on the assumption of positive chemotactic influences exerted by the cells of Schwann of the peripheral part. This assumption is merely hypothetical. An anatomical conception of the centrifugal orientation based upon my experiments seems more satisfactory, assuming that the protoplasmic syncytium of Schwann growing out from the peripheral part of a divided nerve, branching in the scar may receive the axis cylinders coming from the central part and serve as a guidance for them into the peripheral segment. In no case did I observe axis cylinders forming inside or growing out from the incubated nerve fibers. The fact that axis cylinders grow out into the plasma from pieces of the central nervous system containing ganglia

[9] Forssman, J., Beitr. z. path. Anat. u. z. allg. Path., 1898, xxiv, 56; 1900, xxvii, 407.
[10] Cajal, Studien über Nerveregeneration, Leipzig, 1908.

cells, is an additional argument in favor of the monogenetic theory of the regeneration of nerves.

From a morphological standpoint the study of the grown out syncytium of Schwann in the plasma presents some features of interest. The study of the structure of the protoplasm of Schwann is a difficult task, and it is only in the last few years that the work of Nageotte has elucidated its syncytial nature. The protoplasm growing out from the cells of Schwann in the plasma in the first stages of the Wallerian degeneration shows a tendency to form a syncytial network. In the protoplasmic structures growing out in the later stages of Wallerian degeneration the liability to branch and anastomose is not so pronounced; the straight parallel rows of cells are similar to the so called bands of von Büngner.[11] The bands of von Büngner have been interpreted by this investigator and several others after him as the first signs of a differentiation of an axis cylinder out of the multiplied and coalesced cells of Schwann in a degenerating nerve. The errors of this interpretation, however, have been shown by Cajal[10] and Nageotte.[12] My photographs of the syncytium of Schwann growing in plasma, and showing the morphological likeness between the structures and axis cylinders, give an additional demonstration of the error. It is interesting to note the corresponding appearance of a syncytium of Schwann and syncytial neuroglia growing in plasma. There is very little difference between the pictures, and this is not surprising, when we take into consideration the common origin of these tissues in the early fetal life of the organism.

CONCLUSIONS.

1. The Wallerian degeneration occurring in peripheral nerves by incubation in Ringer solution and serum does not occur in plasma.

2. Peripheral nerves incubated in plasma give rise to no growth. The same is true of peripheral nerves in a Wallerian degeneration up to the 4th day.

3. Peripheral nerves in Wallerian degeneration from the 5th day give rise to a growth of the syncytium of Schwann. In cultures from

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[11] von Büngner, O., *Beitr. z. path. Anat. u. z. allg. Path.*, 1891, x, 321.

[12] Nageotte, *Compt. rend. Soc. de biol.*, 1911, lxx, 861.

later stages there is a progressive growth of the same structure. It is evident that the proliferation of the cells of Schwann is directly produced by the degeneration of the axis cylinder and its myelin sheath.

4. In no case was growth of axis cylinders observed.

5. The growth of the syncytium of Schwann from degenerating nerves affords a basis for an anatomical conception of the centrifugal orientation of growing axis cylinders in regeneration.

6. Morphologically there is a striking resemblance between the syncytium of Schwann and neuroglia growing in plasma.

EXPLANATION OF PLATES.[13]

PLATE 34.

FIGS. 1 AND 2. Microphotographs of nerve fibers from the sciatic nerve of a rabbit, incubated in plasma for 12 days. Hardened in formalin and stained with hematoxylin. Fig. 1, low power magnification; Fig. 2, high power magnification. There is no trace of Wallerian degeneration.

FIG. 3. Microphotograph of protoplasmic filaments (syncytium of Schwann) emerging into the plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 5 days after section. Hardened after 6 days of incubation and stained with hematoxylin.

In the center of the picture a fiber is seen including a nucleus of Schwann and three myelin (fatty) globules. Several connective tissue cells are also seen.

PLATE 35.

FIG. 4. Camera lucida drawing of framework-like syncytium grown out from the cut end of a nerve segment removed from the peripheral part of a divided nerve, 5 days after section and incubated in plasma for 6 days. Stained with hematoxylin.

The nuclei of Schwann are surrounded by myelin globules.

PLATE 36.

FIG. 5. Camera lucida drawing of branching protoplasmic filaments grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 6 days after section. Incubated for 5 days and stained with hematoxylin.

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[13] Dr. O. Berner, of the Anatomical Institute, Christiania, kindly made some of the microphotographs, Dr. Platou a few of the photographs.

PLATE 37.

FIG. 6. Microphotograph of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 6 days after section. Incubated for 9 days. Cajal silver impregnation. Low power magnification.

FIG. 7. Camera lucida drawing from the same preparation. The length of the fibers is about 350 microns.

Note the varicosities produced by nuclei.

PLATE 38.

FIGS. 8 AND 9. Microphotographs of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 13 days after section. Incubated for 5 days and stained with hematoxylin. Fig. 8, low power magnification; Fig. 9, high power magnification.

PLATE 39.

FIGS. 10 AND 11. Microphotographs of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 19 days after section. Incubated for 6 days and stained with hematoxylin. Fig. 10, low power magnification, showing the whole bunch of filaments; Fig. 11, high power magnification, giving details from the same preparation.

PLATE 40.

FIG. 12. Microphotograph of the branching and anastomosing syncytium grown in plasma, from a piece of cerebellum of a young dog. Culture 2 days old stained with hematoxylin. High power magnification (1,900 diameters).

PLATE 41.

FIG. 13. Microphotographs (A) focused on three different planes of branching and anastomosing syncytium grown out from a big neuroglia cell of a piece of cat cerebellum. Culture 3 days old. Cajal silver impregnation. B is a drawing of the same structures.

Fig. 1.   Fig. 2.

Fig. 3.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

FIG. 4.
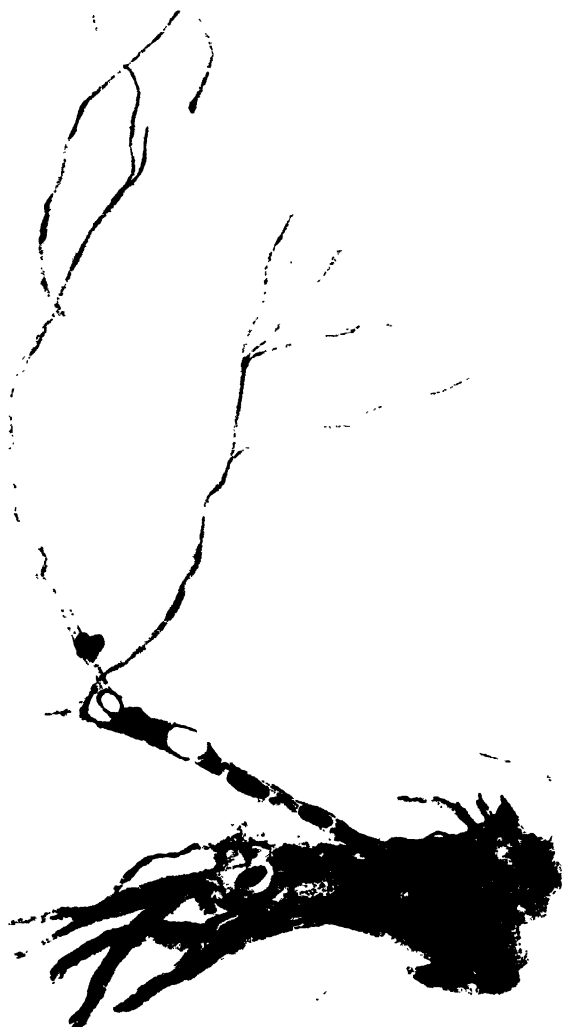
Fig. 5.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

Fig. 6.

Fig 7.

FIG. 8.

FIG. 9.

Fig. 10.

Fig. 11.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

FIG. 12.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

FIG. 13.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

THE RELATIVE EFFICIENCY OF VARIOUS PARTS OF THE SPECTRUM FOR THE HELIOTROPIC REACTIONS OF ANIMALS AND PLANTS.

SECOND COMMUNICATION.[1]

BY JACQUES LOEB AND HARDOLPH WASTENEYS.

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I.

In 1869 Paul Bert made experiments on the effect of different parts of the spectrum on Daphnia to find out whether the vision of these crustaceans is comparable to that of man. He found that the animals "accouraient beaucoup plus rapidement au jaune ou au vert qu' à toute autre couleur," and concluded from this that the green and yellow rays which appear comparatively bright to us appear also brightest to these animals.[2]

At that time biologists were generally under the influence of the anthropomorphic viewpoint and did not hesitate to interpret the reactions of animals on the basis of human analogies; although it must have occurred to as clear a thinker as Bert that there is no *a priori* reason for assuming that human beings when put into a spectrum must gather in the green or yellow; and experiments in this direction were lacking. This anthropomorphic viewpoint appeared still more plainly in the writings of Graber,[3] who experimented with animals which were kept in a box illuminated from above, one-half of which was covered with red, the other with blue glass. He found that animals which prefer darkness to light gather under the red; and ani-

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[1] First Communication, Jour. Exp. Zoöl., 1915, 19, 23. See also Loeb and Wasteneys, Proc. Nat. Acad. Sc., 1915, 1, 44; Science, 1915, 41, 328.

[2] Paul Bert. Arch. de Physiol., 1869, 2, 547.

[3] Graber. Grundlinien zur Erforschung des Helligkeits- und Farbensinnes der Tiere. Prag, 1884.

mals which prefer light to darkness gather under the blue glass. This led him to enunciate the law that animals which are 'fond' of light are also 'fond' of the blue; and animals which are 'fond' of the darkness are also 'fond' of the red. We see again the tacit assumption that animals which collect under blue glass do so because they are 'fond' of this type of light, while animals which collect under the red light do so because they are 'fond' of this type of light.

The field of animal reactions received a different interpretation by Loeb,[4] who showed that these results can be explained on a purely objective basis without our ascribing to lower organisms sensations the existence of which we can neither prove nor disprove. Loeb showed that the phenomena observed by Bert, Graber, and others can be explained on the assumption that the light automatically orients the animals or determines the direction in which they move, there being two classes of animals, one class being automatically compelled to move to the source of light, the other being compelled to move in the reverse direction; and he pointed out that this phenomenon is the same as the heliotropic reaction in plants, the stem of plants bending to the source of light, the roots bending away from it; or the swarmspores of algae moving to or from the light. Accordingly he designated the animals going to the light as positively heliotropic, those going away from the light as negatively heliotropic.

As this bending effect in the plant is a purely automatic orientation of the plant, brought about through the influence of the light, so in the animals we are, according to Loeb's theory, dealing only with an orienting effect of the light for the explanation of which merely physicochemical conditions are adequate; without our being compelled to introduce hypothetical sensations as a necessary link in the mechanism. This purely mechanistic conception of the motions of animals to or from the light has recently received a new support by the invention of heliotropic machines by Mr. John Hays Hammond, Jr., in which the two retinas are replaced by selenium wire, these machines following a lantern in the dark in the same way as a positively heliotropic animal.

[4] Loeb, J. Der Heliotropismus der Tiere und seine Uebereinstimmung mit dem Heliotropismus der Pflanzen. Würzburg, 1890. Sitzungsber. d. Würzburger physikal-med. Gesellsch., Jan. 1888.

It was easy to interpret the phenomena found by Graber from this heliotropic viewpoint. The botanists had long ago shown that positively heliotropic plants bend readily to the light when behind a blue screen, while they do not do so or only very slowly when behind a red screen; from which they concluded that the rays going through a blue screen had a higher heliotropic efficiency than the rays passing through a red screen.

If the animals were, as Loeb stated, merely positively or negatively heliotropic the light going through blue glass should act like more intense light than that going through red glass, and hence negatively heliotropic animals should gather in the red, positively heliotropic animals in the blue. He could show by a series of experiments that this statement was correct. In this way, purely objective methods and explanations were given for the arbitrary assumption of Graber and the other anthropomorphic biologists that animals moved to or from the light because they were 'fond' of light.

The advantage of this change in viewpoint lies in the fact that it opened this field to the methods of exact experiments and measurements without which no progress is possible; while the attempt to explain reactions by a hypothetical 'fondness' of animals for light or by hypothetical light sensations barred the way to the exact type of investigation.

Recently, however, the old anthropomorphic viewpoint has been resumed by the ophthalmologist Hess,[5] who has tried to show that all the animals from fish downward suffer from a visual deficiency, namely total color blindness. As a criterion for the presence or absence of color sensations Hess uses (very arbitrarily in our opinion) the heliotropic reactions of animals. Thus in 1909 he confirmed Bert's observation that Daphnia collect in the yellow-green part of the spectrum but gave it a different interpretation. By calling attention to the fact that the yellowish-green; which is heliotropically most efficient for Daphnia, appears brightest to the human afflicted with total color blindness, he thinks he has proved that Daphnia is also totally color blind.

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[5] Hess. Gesichtssinn. Handb. d. vergleich. Physiol., 1913, 4, 555.

The number of objections to this kind of reasoning is considerable.[6]

Nobody has yet proved that the heliotropic reactions of animals are determined or even accompanied by any sensations of brightness and it is difficult to see how such a proof can ever be furnished. It is plainly unwarranted to assume that every motion of animals induced by light is accompanied by or is the expression of sensations of brightness or of color. The excised iris of the shark (and of other animals) contracts under the influence of illumination and Magnus has shown that the yellowish-green part of the spectrum is most efficient in this case. It would be arbitrary, to say the least, to state that the excised iris has sensations of brightness and that these sensations make it contract; and yet it is difficult to see why such an assumption should be more arbitrary than a similar assumption in the case of the flagellate Chlamydomonas or the heliotropic larvae of Balanus. One wonders also whether we are supposed to assume that Hammond's heliotropic machines are guided by sensations of brightness or of color.

The assumption of Hess might be given some consideration if it could be shown that totally color blind human beings are positively heliotropic, i.e., are irresistibly drawn to the source of light; but nobody has ever heard of such a case. The human being is the only one about whose sensations we have definite knowledge and as long as we are unable to prove for the human a connection between positive heliotropism and the sensations of brightness we have no right to take such a connection for granted in the lower animals.

One wonders also what interpretation is to be put on other tropisms, such as galvanotropism or geotropism, if we accept the validity of Hess's viewpoint, since it is only logical to treat all the tropisms from the same general viewpoint. What sensations are aroused in a Paramaecium which is forced to swim to the cathode under the influence of the galvanic current, or in a Palaemonetes which is forced to swim or walk to the anode?

Since Hess starts with an arbitrary assumption, namely that the heliotropism of lower animals is due to their sensations of brightness

[6] An excellent criticism of Hess's ideas and experiments has been given by W. F. Ewald, Arch. f. Entwcklngsmech., 1915, 37, 581. We are using some of his arguments in this paper.

and that they are totally color blind, it is not unexpected to see him come into conflict with facts in more than one direction. V. Frisch[7] has shown by experiments which appear to us conclusive that bees (which are also positively heliotropic and which according to Hess are totally color blind) can be trained to go to yellow or blue cardboards distributed among similar cardboards of different shades of gray; while they can not be trained to go to definite shades of gray under similar conditions. Even in Daphnia v. Frisch and Kupelwieser,[8] and Ewald[9] have been able to demonstrate selective effects of wave lengths different from those found in the totally color blind human.

A second conflict between Hess's view and reality is due to the fact that the most efficient part of the visible spectrum is not the same for all heliotropic organisms. It is known through Blaauw's experiments that the heliotropic curvatures of the seedling of oats are produced most rapidly in the blue part of the carbon arc spectrum. This should force Hess either to the conclusion that the seedlings of oats do not suffer from total color blindness, since the most efficient part of the spectrum for the totally color blind is in the yellowish-green; or to the assumption that only plants are heliotropic, but that animals which show the same reactions to light are not heliotropic. Hess chooses the second alternative by stating that plants are heliotropic, while animals are 'lamprotropic' ($\lambda\alpha\mu\pi\rho\dot{o}s$ = bright),[10] i.e., in plants the heliotropic curvature occurs purely automatically, while animals bend or move to the source of light because it is 'bright.' It would be difficult to invent a nicer example of reasoning in a circle; since Hess's assumption that animals have the sensation of brightness is based upon the fact that they move to the light.

Yet we will try to follow Hess even into this circle and select a case already mentioned in a previous note[11] and to which we shall return in this paper, namely the case of two green flagellates, Euglena viridis and Chlamydomonas pisiformis, which are strongly heliotro-

[7] v. Frisch. Der Farbensinn und Formensinn der Biene, Zool. Jahrb., 1914, 35, 1, Abt. f. allg. Zool. u. Physiol.

[8] v. Frisch and Kupelwieser. Biol. Centralbl., 1913, 33, 517.

[9] Ewald. Ztschr. f. Psychol. u. Physiol. d. Sinnesorg., 1914, 48, Abt. 2, 285.

[10] Hess, loc. cit., pp. 708 and 709.

[11] Science, 1915, 41, 328.

pic, but, being unicellular organisms, of course have no eyes. For Chlamydomonas the place of greatest efficiency in the spectrum is in the region of yellowish-green, for Euglena it is in the blue. If we follow Hess we must logically conclude from this that Chlamydomonas suffers from total color blindness (although it has no eyes), that it is not heliotropic but 'lamprotropic,' and that it is an animal; while its cousin Euglena has either a highly developed color sense or is heliotropic and is a plant.

Hess[12] thinks it is inconsistent for Loeb to deny the justification of the assumption that all heliotropic animals are totally color blind and at the same time to state that phenomena of heliotropism are identical in animals and plants. Hess overlooks the fact that the two statements rest on an entirely different basis. The statement that positively heliotropic animals go to the light because they are totally color blind is as we have seen not a fact but an unnecessary and arbitrary assumption which is in conflict with the facts and not even justifiable on the basis of mere analogy, since totally color blind humans are not positively heliotropic. The fact that the region which is brightest to the totally color blind human is at the same time most efficient in certain heliotropic animals admits or demands, as we shall see, an entirely different interpretation.

On the other hand, the statement that heliotropic reactions in animals and plants are identical is merely the expression of the actual observations. Thus Loeb has been able to show that sessile heliotropic animals react to one-sided illumination just like sessile plants, namely by bending towards the source of light until the axis of symmetry of their photosensitive organs goes through the source of light (provided only one source of light is given); while movable plant organs, e.g., the swarmspores of algae move to or from the source of light and collect on the side of the light (or on the opposite side) just as do motile heliotropic animals. For the heliotropic reactions of both animals and plants the validity of the law of Bunsen and Roscoe has been proved[13] and the sense of heliotropic reactions in both groups can be reversed by similar means.[14] It would be artificial to

[12] Loc. cit., p. 709.

[13] Loeb and Ewald. Centralbl. f. Physiol., 1914, 27, 1165; Ewald, Ztschr. f. Psychol. u. Physiol. d. Sinnesorg., 1914, 48, Abt. 2, 285.

[14] Loeb. Arch. f. d. ges. Physiol., 1906, 115, 564.

state that because the ones are termed animals and the others plants the identical phenomena in both must be different. Such a view might have been considered at the time of Linné, but today we know that the mechanism of life phenomena in animals and plants is essentially the same. While modern biology, especially since Claude Bernard and Hoppe-Seyler, has tried to establish the essential identity of life phenomena in plants and animals, Hess apparently expects biologists to overlook the progress made in biology and return to the Linnéan viewpoint. In order to maintain his artificial barrier between animals and plants he insists that the wave length which is most efficient in the heliotropic reactions in plants is different from the one most efficient in animals. But even if this were a fact, it would not justify his assumption, since the theory of heliotropism only states that organisms are automatically oriented by the light so that symmetrical elements of their photosensitive surface are struck at the same angle by the light (or that symmetrical elements receive an equal amount of illumination during a properly chosen unit of time). Whether in one case the yellowish-green, in another the blue light is more efficient is secondary.

As a matter of fact, there are heliotropic animals for which the blue rays are as efficient as they are for plants; and there are unicellular organisms, for which the optimum lies in different parts of the spectrum.

From the viewpoint of objective science we accept the fact that in some heliotropic organisms the place of highest efficiency is in that region of the spectrum which for the totally color blind is the brightest, but on this we put a different interpretation, namely the following. The sensations of brightness in the totally color blind human are determined by the rapidity with which visual purple is bleached by the light. The region in the yellowish-green in the carbon arc spectrum appears brightest to the totally color blind human because this region $\lambda = 526 \mu\mu$ has according to Trendelenburg the greatest bleaching effect. Assuming that heliotropic reactions are also due to a photochemical effect, the fact that in certain organisms the region not far from $\lambda = 526 \mu\mu$ is the most efficient in calling forth heliotropism means simply that the photosensitive substance responsible for the heliotropic reaction in these organisms has one peculiarity in

common with the visual purple, namely that it is also most sensitive to a region not too remote from $\lambda = 526 \, \mu\mu$; the two substances may possibly be identical, but this would require a definite proof. The fact that the optimal effect for other organisms lies in the region of blue would indicate that the photosensitive substance in these animals is in all probability different from visual purple.   If the effect of light in causing heliotropic reactions were other than chemical we still should be compelled to find a physicochemical and not a psychological explanation for the different heliotropic efficiency of different wave lengths.

The question to which we intend to confine ourselves in this paper is a very simple one, namely: Is it true that a sharp line of demarcation exists between animals and plants in that sense that for the heliotropic reactions of plants the blue is most effective, while for the heliotropic reactions of all animals a region in the yellowish-green is the most efficient, as Hess claims?   In this paper we shall deal with motile organisms, namely first the two unicellular green organisms Euglena and Chlamydomonas and the larvae of two animal forms, of the annelid Arenicola and of the crustacean Balanus eburneus.

II. Methods.

When we wish to determine where the most efficient spot of the spectrum for freely moving animals lies, we must realize that in order to get reliable results we must work with organisms which are both very small and very sensitive to light.   The organisms must be small so that a large number can be crowded into a narrow region of the spectrum; if this condition is not fulfilled it is extremely difficult if not impossible to make statements concerning the relative efficiency of the different parts of the spectrum which are of sufficient accuracy. Thus attempts to determine the most efficient spot for the heliotropic efficiency of the spectrum for young fish or larger insects can only yield crude approximations.   The second condition is that the animals must be very sensitive to light; since Loeb has shown in former papers that only in the case of extreme sensitiveness will the animals go directly to the source of light, while if the sensitiveness is small the animals may go in very irregular paths although the sum total of the

motions towards the source of light will prevail. It would be impossible to get a definite result with animals of this kind. Thus experiments on the relative efficiency of different parts of the spectrum with young fish or other animals which are only moderately sensitive are very unreliable.

When we are dealing with positively heliotropic animals distributed in an oblong trough exposed to a carbon arc spectrum the animals will move towards the source of light independently of the nature of the rays by which they are struck; provided intensity and frequency of the waves are above the threshold of heliotropic efficiency. If the animals are evenly distributed in the trough at the beginning of the experiment they will all move towards the source of light and the result should be that at the end of the experiment the animals should all gather equally at the front wall of the trough and their density should be the same on this front wall in the violet-blue and green provided that the rays are sufficiently effective. On this basis it should be difficult to tell whether the blue or the green is more efficient. Since, however, some scattering of light occurs from the surface of the animals, some blue light will also reach the animals in the green and vice versa. In this way a comparatively denser gathering of animals may occur in that part of the spectrum which is more effective. It is obvious, however, that this method is not very exact since in an aquarium with animals the scattered light from one part of the spectrum can chiefly reach only those individuals which are not too far from this spot.

The second method consisted in the comparison of the relative efficiency of two narrow parts of the spectrum. It was described briefly in a former note.

A carbon arc spectrum, about from 18 to 23 cm. wide, was thrown on a black screen SS (see fig. 1) with two slits a and b in the two different parts of the spectrum which were to be compared in regard to their heliotropic efficiency. The two beams of light passing through the slits are reflected by the two mirrors M and M_1 into the square glass trough in such a way as to strike the same region g of the back wall of the trough. The glass trough is surrounded by black paper except at R and R_1, where the two beams of light enter from the mir-

rors. Before the experiment begins, all the organisms are collected in the spot g with the aid of an incandescent lamp. As soon as the spectrum is turned on, these organisms are simultaneously exposed to two different beams of light which come from the two mirrors M and M_1. When one type of light, e.g., that from M, is much more efficient than the other coming from M_1, practically all the organisms are oriented by the light from M and move towards this mirror, collecting in the region R. When the relative efficiency of the two types
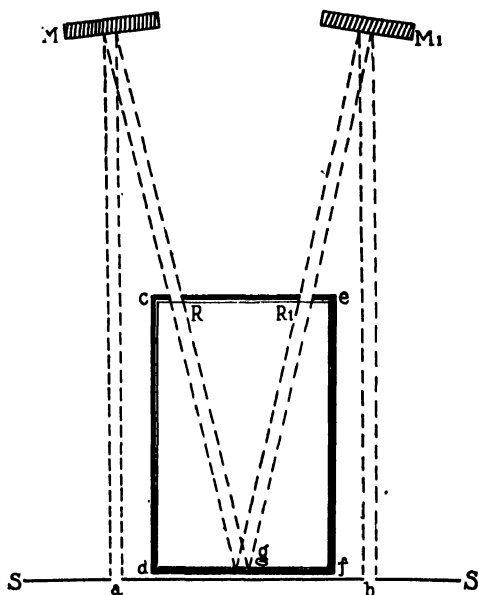
1.

of light is almost equal the organisms move in almost equal numbers to R and R_1. By using as a standard of comparison the same region of the spectrum and successively altering the position of the other slit in the spectrum we were able to ascertain with accuracy the relative efficiency of the different parts of the spectrum for the two forms of organisms. When the two parts of the spectrum which are to be compared are very close to each other it is necessary to deflect the beams with the aid of deflecting prisms, before they reach the two mirrors.

III. The Distribution of Organisms in the Carbon Arc Spectrum.

The spectrum used was a carbon arc spectrum and its visible part had a width varying from 18 to 23 cm. in different experiments. We used very dense cultures of Euglena viridis and filled the trough with this greenish suspension of Euglena. After an exposure varying in length between 30 and 180 minutes the results were ascertained, and in some cases the trough was photographed. Figure 2 gives the photograph of the trough after 30 minutes' exposure. A very dense
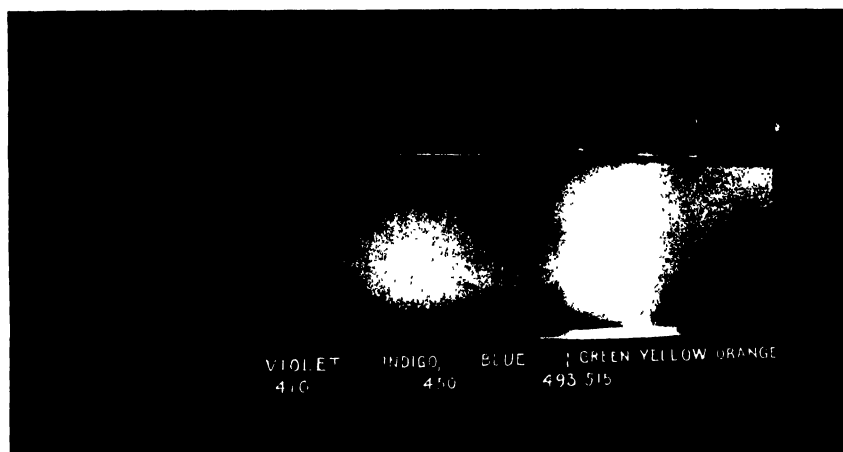
2.

mass of Euglena was gathered at the bottom of the trough in front between violet (410 $\mu\mu$) and green (515 $\mu\mu$). In the photograph this mass is visible as a thick, dark, horizontal streak at the bottom. In addition some vertical streaks of organisms are visible in the blue and indigo. The reader will recognize how difficult it is to ascertain the most efficient wave length by this method. We can only say the blue is the most efficient light and the wave lengths > 515 $\mu\mu$ are practically without orienting effect.

Figure 3 gives a photograph of the distribution of the organisms in another experiment after 3 hours in the same spectrum. This longer exposure gives a slightly better result. The dense gathering at the bottom indicated by a horizontal streak is in the blue between

458 and 506 $\mu\mu$. It ends at about 533 and becomes very faint at 452 $\mu\mu$. The vertical streaks on the wall, indicating the sticking of the organisms to the front wall, occur again in the blue. These experiments permit us to draw only the conclusion that the blue is more effective than the green, yellow, red, indigo, and violet; but they do not permit a more definite statement.

Engelmann states that he found a strong gathering of Euglena in the blue between 470 and 490 $\mu\mu$ in a spectrum.[15]

When we made similar experiments with Chlamydomonas pisiformis, which is also a chlorophyll-bearing unicellular organism like

| VIOLET | INDIGO | | BLUE | GREEN | | YELLOW | ORANGE |
| WAVE LENGTH $\mu\mu$ | 452 | 458 | | 506 | 533 | | |
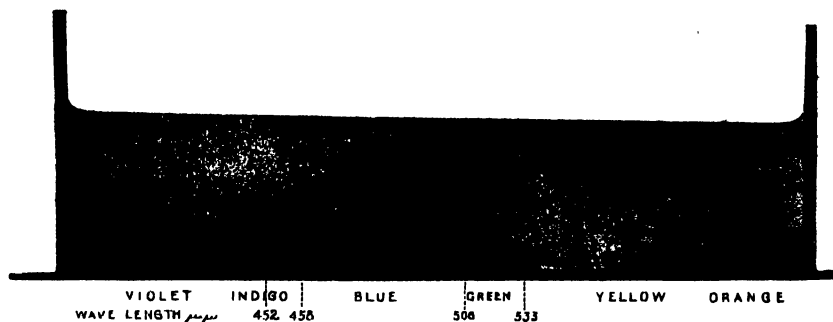
3.

Euglena, we noticed that the gathering went much farther towards the yellow ending at about $\lambda = 560$ or $570\ \mu\mu$. The region of maximal gathering seemed to be at about $\lambda = 520\ \mu\mu$. A similar result had previously been obtained with Chlamydomonas by Loeb and Maxwell.[16]

The method is still less definite with larger and rapidly moving animals, and yet it is mainly by such experiments that Hess tried to prove that the most efficient part of the spectrum for heliotropic animals is identical with that which appears brightest to the totally color blind.

[15] Engelmann. Arch. f. d. ges. Physiol., 1882, 29, 387.
[16] Loeb and Maxwell. Univ. Cal. Pub., 1910, Physiol., 3, 195.

IV. Experiments with the Two-Beams Method.

With the aid of two slits (fig. 1) two narrow strips of the spectrum were cut out. Their width was such that in the green part of the spectrum the difference in the wave length of the extreme rays that passed through was about 10 $\mu\mu$. With the aid of prisms and mirrors these two parts of the spectrum were made to converge to one spot in the trough where the organisms had previously been collected. It was ascertained which of the two beams of light was more powerful.

In detail the experiments were as follows. The trough was surrounded with black cardboard in which there was one opening at that spot where it was intended the animals should collect. Then an incandescent lamp was turned on in front of this opening which caused all the organisms to collect at that spot of the trough. When this happened, the spectrum was turned on and the incandescent lamp turned off and the animals were exposed to the two beams of light a and b selected for comparison, after another black cardboard with openings at R and R_1, to let the two beams of light pass, had been put over the box, and the first cardboard enclosing the animals had been removed. The organisms collected at g were now under the influence of these two beams coming from different directions. As stated before, they moved towards R and R_1 according to the selective efficiency of the two beams of light. The readings were taken after from 15 minutes to 3 hours or more to make sure that the results were permanent. Before a new experiment was made the organisms were all scattered equally again in the trough. Fresh organisms were used every day.

As stated before, one of the two parts of the spectrum was the same in a group of experiments while the other changed in successive experiments throughout the spectrum. In figure 4 the results of 21 experiments with Euglena are plotted. The part of the spectrum which was stationary was situated at about 470 $\mu\mu$ which previous experiments had led us to believe was the most efficient wave length. The different degrees of blackness indicate the denseness of the gathering; the more animals gathered in one spot the darker the oblong representing the experiment. We notice that the oblongs at the region 470 $\mu\mu$ are with two exceptions much darker than all those at other wave lengths. These results indicate that the greatest efficiency

is possessed by the rays between 460 and 490 $\mu\mu$ for Euglena viridis. The total of all experiments is represented in diagram II, figure 7, where the distribution of this efficiency through the carbon arc spec-

Euglena viridis.
Wave lengths in $\mu\mu$.
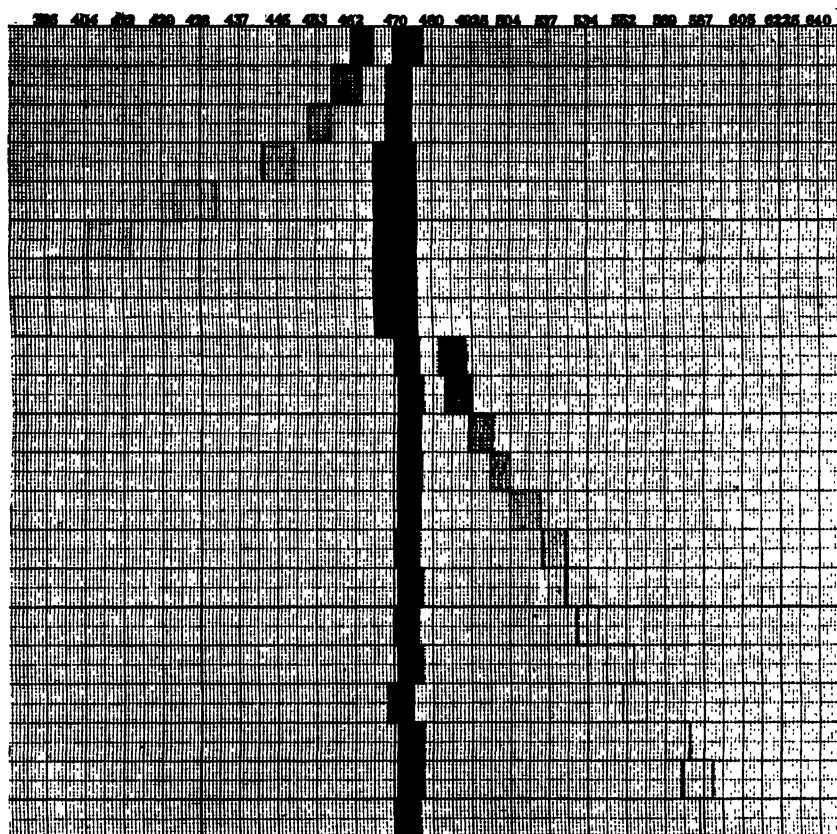
4.

trum is plotted for this form.   The greatest efficiency is indicated by the greatest blackness, the greater. blackness indicating the denser gathering.

The results with Chlamydomonas were entirely different.   In figure 5, the region between 470 and 480 $\mu\mu$ was again constant in each

determination, while the region compared with this varied in each experiment. It is obvious that in contradistinction to the experiments on Euglena the region between 460 and 480 $\mu\mu$ was less efficient than the region from 490 to almost 560 $\mu\mu$.

We, therefore, started another series of determinations in which the region about 534 $\mu\mu$ was constant (fig. 6). We now found that this

Chlamydomonas pisiformis. Dill.
Wave lengths in $\mu\mu$.
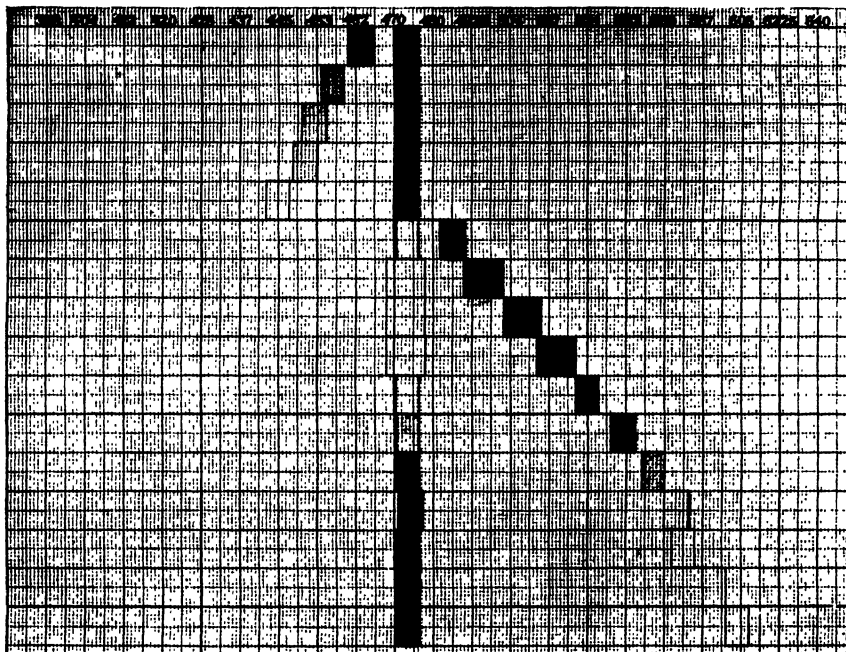
5.

region was more efficient than any other region in the visible spectrum. The experiments show that the maximal efficiency lies for Chlamydomonas, approximately in that part of the spectrum which appears brightest to the totally color blind human.

In the same way long series of experiments were made with the newly hatched larvae of Arenicola, an annelid. The experiments in this form suffer from the difficulty that the larvae have a tendency

to stick to the glass walls of the trough. We found that if we keep them in the dark before using them they are more sensitive to light and less liable to stick so soon, and such animals gave clearer results. The most efficient part of the spectrum was situated in the bluish-

Chlamydomonas pisiformis. Dill.
Wave lengths in $\mu\mu$.
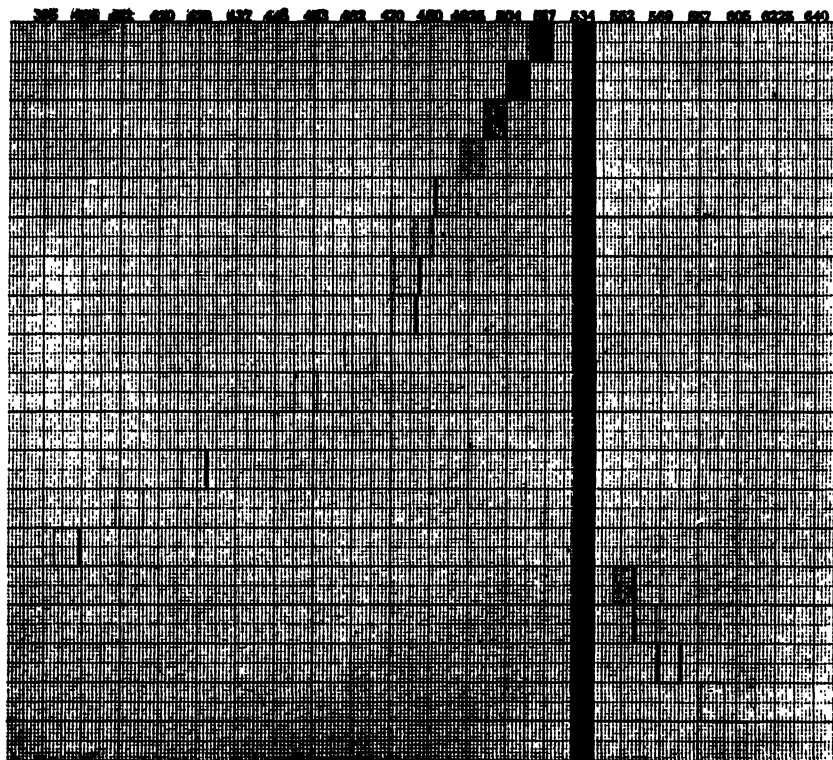
6.

green in the region of about $\lambda = 495 \ \mu\mu$. And finally, experiments with this method on the larvae of Balanus eburneus yielded the result that the most efficient part .of the spectrum lies between $\lambda = 560$ and $\lambda = 578 \ \mu\mu$.[17]

[17] These results agree with previous observations by Loeb and Maxwell, Univ. Cal. Pub., 1910, Physiol., 3, 195.

The relative efficiency of the different parts of the carbon arc spectrum for different organisms is plotted in figure 7. The upper line gives the wave length and below is found the relative efficiency of the various wave lengths as revealed by the two-beams method for Eudendrium ramosum, Euglena viridis, larvae of Arenicola, Chlamydomonas pisiformis, and larvae of Balanus; the greater darkness indicating the greater efficiency. The two most striking facts to us are, first, that there are animals for which the most efficient part of the spectrum is in the blue, namely Eudendrium and the larvae of Arenicola; and that among the flagellates, Chlamydomonas is most sensitive to yellowish-green, while the closely related Euglena is most sensitive to the blue.

The second striking fact is that the place of greatest efficiency does not seem absolutely identical in the organisms of the same group. In the line VI of figure 7 is represented the relative brightness of the various parts of the spectrum for the totally color blind human after Helmholtz. A comparison with the relative efficiency of the various wave lengths for Balanus larvae and Chlamydomonas (IV and V, fig. 7) shows that the maximal efficiency is not entirely identical in all three cases. We are not prepared to state whether this is entirely due to the inadequacy of the methods.

DISCUSSION AND SUMMARY OF RESULTS.

1. As stated in the previous papers, the validity of the Bunsen-Roscoe law for the heliotropic reactions of some (and possibly many or all) organisms suggests that these reactions are due to a chemical action of the light. There seem to exist two types of heliotropic substances (or elements), one with a maximum of sensitiveness in the yellow-green region, and the second with a maximum of sensitiveness in the blue.

2. It would be wrong to state that the one type of photosensitive substances is found exclusively in plants and the other exclusively in animals. As a matter of fact, our experiments have shown that the animals Eudendrium ramosum and (the larvae of) Arenicola are most sensitive to blue light, which is also most efficient for the seedlings of the plant Avena (according to Blaauw); while the larvae of

Balanus, Daphnia, and probably many other animals are most sensitive to the yellow-green or yellow part of the spectrum. Of the two green flagellates, Euglena viridis and Chlamydomonas pisiformis,

Wave lengths in $\mu\mu$.

392 400 407.5 416 424 432.5 441 449 457.5 466 474 486 495 510 525 543 560 578 596 613 631

I   EUDENDRIUM RAMOSUM

II EUGLENA VIRIDIS

III ARENICOLA LARVAE

IV CHLAMYDOMONAS PISIFORMIS

V   BALANUS LARVAE

VI TOTALLY COLOR BLIND HUMAN

VIOLET   INDIGO   BLUE   GREEN   YELLOW   ORANGE
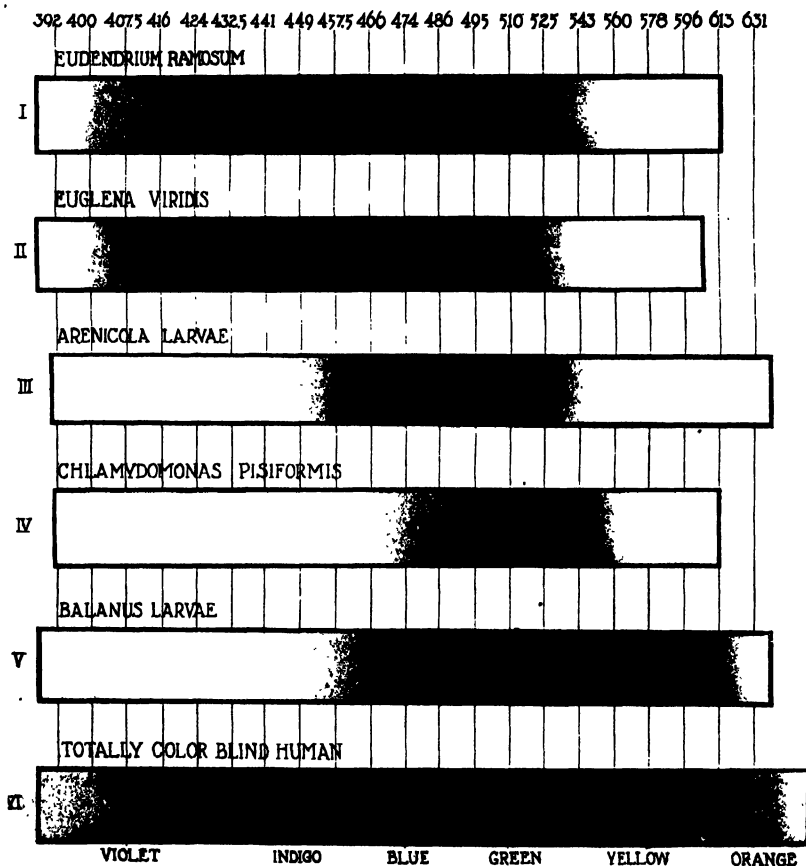
7.

the former is most sensitive to blue, the latter to greenish-yellow. The two groups of photosensitive substances (or elements) are, therefore, distributed independently of the boundaries between animals and plants. It is quite possible, however, that plants are more gener-

ally sensitive to the blue rays of the spectrum, while among animals those may prevail that are more sensitive to yellowish-green or yellow.

3. Table I states the wave lengths in the carbon arc spectrum for which the different organisms investigated by us are most sensitive.

Visual purple is bleached most rapidly by light of the wave length of about 530 $\mu\mu$ (according to Trendelenburg). Neither Chlamydomonas nor the larvae of Balanus show their maximal sensitiveness exactly at this point; whether the slight deviation is only due to the

TABLE I.

Name of organism.	Region of greatest heliotropic efficiency.
	$\mu\mu$
Eudendrium ramosum...................	460–480
Euglena viridis........................	460–490
Larvae of Arenicola....................	about 495
Chlamydomonas pisiformis..............	about 535
Larvae of Balanus eburneus.............	560–578

imperfections of the experimental method or due to the fact that the photosensitive substance is not identical with visual purple can not be decided on the basis of the material which is at present available.

We find likewise that those organisms which react best to the blue part of the spectrum do not all have their greatest sensitiveness in the same spot of the blue. Thus the seedlings of oats are most sensitive to a region of $\lambda = 466$ $\mu\mu$, the animal Eudendrium and the flagellate Euglena for a region near $\lambda = 460$ to 490 $\mu\mu$, while the larvae of Arenicola are most sensitive to $\lambda = 495$ $\mu\mu$.

THE RATE OF OXIDATIONS IN REVERSED ARTIFICIAL PARTHENOGENESIS.

By HARDOLPH WASTENEYS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In 1913 Loeb[1] showed that by suitable treatment unfertilized eggs of the sea urchin *Arbacia*, in which development had been artificially initiated, could be caused to revert to their original inactive condition. This was accomplished by placing the eggs in solutions of sodium cyanide or chloral hydrate immediately after a preliminary treatment with butyric acid or an alkali or with alkaline hypertonic solution.

If the treatment with butyric acid or alkali only was applied, or if, after this treatment, the eggs were placed in the sodium cyanide or chloral hydrate solutions for an insufficient length of time, they would disintegrate on transference to normal sea water. On the other hand, eggs which had received a sufficiently long treatment with sodium cyanide or chloral hydrate, on transference to normal sea water, remained intact and could be either fertilized or, in some cases, induced to develop again by the usual methods of artificial parthenogenesis. Clearly, a reversal of the effects induced by the alkali or acid treatment had taken place.

The chloral hydrate and sodium cyanide treatments, as Loeb pointed out, cause the reversal of the induction of development by suppressing the developmental changes due to the alkali or acid treatment.

Until more is known as to the nature of the changes taking place in the egg on membrane formation, it is difficult to form any idea as to the nature of the reaction whose rate is decreased, but in view of the marked changes in the rate of oxidations in the egg coincident with fertilization or artificial membrane production, it seemed of

[1] Loeb, J., *Arch. Entwcklngsmechn. Organ.*, 1914, xxxviii, 277.

interest to follow the course of the oxidations during the process of reversion described by Loeb.

In the following experiments the rate of oxidations was measured at different stages in the method used by Loeb,[2] and the results show clearly that after the reversal which he described the oxidations indeed continue at the normal rate existing in the unfertilized, untreated egg.

I. Method.

The method used in the determination of the rate of oxygen consumption was that of Siebeck.[3] This method possesses the great advantage that only relatively small amounts of egg suspension are required for a determination, and also that the rate can be ascertained at any desired moment during a determination. The readings are made by means of a water manometer, and the size of the apparatus is so arranged that 1 mm. pressure on the water manometer corresponds almost exactly to an oxygen consumption of 1 c. mm.

Heilbrunn[4] has recently objected that results by this method are not reliable, in that substances diffusing out of the egg into the solution cause a lowering of its solubility for oxygen, thus yielding a low result. Apart from the fact that an absolute value for the consumption of oxygen is neither necessary nor desired in such experiments, this source of error, though it undoubtedly exists, is shown to be negligible by means of simple controls suggested by the author of the method and readily carried out with *Arbacia* eggs.

In order to give some idea of the method of manipulation, a typical experiment will be briefly described.

A suspension of *Arbacia* eggs, after thorough mixing, was divided into six equal parts. Two of these parts were used for the control determination of oxygen consumption by the unfertilized eggs. The controls having been set aside, the remaining four portions were mixed, treated for $2\frac{1}{2}$ minutes with butyric acid (2.0 cc. 0.1 N acid to 50 cc. sea water), and immediately transferred to 50 cc. sea water

[2] Loeb, *Science*, 1913, xxxviii, 749; *Arch. Entwcklngsmechn. Organ.*, 1914, xxxviii, 277.

[3] Siebeck, R.. Abderhalden's *Handb. biochem. Arbeitsmethoden*, 1915, viii, 33.

[4] Heilbrunn, L. V., *Science*, 1915, xlii, 615.

+ 0.4 cc. 0.1 per cent sodium cyanide solution. This suspension was then divided into two equal portions, each one-third of the original amount of eggs taken. The first of these was washed four times in the sea water + sodium cyanide solution and finally divided into two equal portions, each one-sixth of the original amount, and placed in stoppered flasks. The other portion which had been treated with butyric acid was washed four times in normal sea water, and finally it too was divided into two equal portions. As a result of this manipulation, there were obtained six equal suspensions of eggs; two of these, the controls, had received no treament, two had been treated with butyric acid and subsequently kept in the sea water cyanide mixture, while two had been treated with butyric acid and were subsequently kept in normal sea water in which they disintegrated in the course of a few hours.

Each portion received, as nearly as possible, an equal amount of washing, and, in order to equalize any error due to loss of eggs in the process, even the untreated eggs were subjected to the same washing, so that the rates of oxidation in the six final samples are fairly closely comparable. In order to reduce the volume of the suspension to the amount required for the oxygen determination, viz., 2.5 cc., an attempt was made at first to hasten the process by concentration in a slow speed centrifuge. It was found, however, that even when the greatest care was exercised, the eggs sometimes suffered through this treatment and the expedient was resorted to of allowing the eggs to settle by gravity in a centrifuge tube and decanting the supernatant sea water until the desired volume was obtained. The suspension was then transferred, by means of a wide-nosed pipette, to the flask in which the determination was carried out.

The determinations of oxygen consumption were in all cases carried out at a temperature of 25°C., kept constant to within 0.02°C. No matter what previous treatment the eggs had received, the final measurements were made in sea water in every case.

In all the experiments described in this paper, a similar method of manipulation was used.

II. Oxidations in Reversal.

The rates of oxidation in eggs treated with butyric acid and kept subsequently in sodium cyanide sea water are given in Table I.   On removal from the sodium cyanide solutions, the eggs were, of course, washed and allowed to remain in normal sea water for about 1 hour before proceeding with the oxygen determination.

TABLE I.

No. of experiment	1	2	3	4	5	6	7	8	9	10
	Oxygen consumed, c. mm. per hr.									
Untreated eggs	27	25	30	30	18	28	20	24	10	17
Eggs treated with butyric acid	75	71	62	53	50	49	27	80	17	56
Eggs treated with butyric acid after about 19 hours in sea water + NaCN	53	40	52	29	21	37	21	22	10	17

Table I shows that, with the exception of the first three experiments in which the technique had not been so well developed and also in Experiment 6, the rate of oxidations after about 19 hours in the sodium cyanide solution is nearly the same as for the untreated eggs.

The subsequent behavior of the eggs was followed for at least 1 day further.   It was noted (1) whether the eggs remained normal on removal from the sodium cyanide solution, which was nearly always the case; (2) whether they could be fertilized, this being nearly always possible, a considerable proportion developing to swimming larvæ; and (3) whether they could be made to develop by repeating the treatment with butyric acid followed by hypertonic sea water, as in the ordinary method of artificial parthenogenesis.   In some experiments a large percentage could be caused to develop to swimming larvæ by such treatment indicating a fairly complete reversal of the effects of the first butyric acid treatment, though in other experiments the reversal tested by this means proved less satisfactory.   In such cases, however, this result found its explanation in the figure obtained for oxygen consumption, the reversion of the latter not being complete.   The completeness of the reversal was also tested by simple

treatment of the eggs for about 20 minutes with hypertonic sea water or removal from the sodium cyanide solution. If the eggs could be caused to develop by such treatment it was indicated that the effect of the original butyric acid treatment till persisted and that therefore the reversal was not complete. In two instances a large percentage of the eggs—about 15 per cent—underwent segmentation and developed to swimming larvæ on treatment in this manner. In the remaining experiments none or very few eggs responded to such treatment.

In some of the experiments the eggs which had remained about 19 hours in the sea water and sodium cyanide mixture were subjected again to the butyric acid treatment and their rate of oxidations was determined. Table II gives the result of these determinations.

TABLE II.

No. of experiment..............	6	7	8	9	10
	Oxygen consumed, c. mm. per hr.				
Untreated eggs................................	28	20	24	10	17
Eggs treated with butyric acid...................	49	27	80	17	56
Eggs treated with butyric acid after 19 hours in sea water + NaCN.........................	37	21	22	10	17
Eggs treated with butyric acid after 19 hours in sea water + NaCN, but treated again with butyric acid......................................	31	49	31	19	29

In all cases save in Experiment 6, the rate of oxidations rose again on retreatment with butyric acid, though the original rate was reached only in two instances. These results are further confirmation of the reversal of the effect induced by the first butyric acid treatment.

The rate of oxidations in eggs treated with ammonium hydroxide and kept subsequently in sodium cyanide sea water was also determined. These results are given in Table III. The method of manipulation was similar to that already described for the butyric acid treatment. The eggs remained for 20 minutes in a mixture consisting of 0.3 cc. 0.1 N ammonium hydroxide with 50 cc. sea water, being then transferred directly to the sodium cyanide solution.

TABLE III.

No. of experiment	11	12	13	14	15	16
	Oxygen consumed, c. mm. per hr.					
Untreated eggs	19	18	24	11	16	9
Eggs treated with NH₄OH	26	28	37	49	48	31
Eggs treated with NH₄OH after about 19 hours in sea water + NaCN	28	38	35	15	17	11
Eggs treated with NH₄OH after about 19 hours in sea water + NaCN and treated again with NH₄OH	53	47	61	29	42	21

In the last three experiments the technique employed was better than in the three earlier ones. These results are substantially the same as those obtained in the experiments with the butyric acid treatment. The rate of oxidations, on removal from the cyanide solution, was approximately the original rate and increased again on retreatment with ammonium hydroxide.

After treatment with the alkali and transference to sea water the eggs disintegrated, though after about 19 hours in the sodium cyanide solution, on transference to normal sea water, they remained intact and normal. Such eggs on removal from the cyanide could be fertilized by sperm, and also in most cases caused to develop on retreatment with ammonium hydroxide or butyric acid and hypertonic sea water. When these eggs were placed directly into hypertonic sea water for about 20 minutes, after removal from the sodium cyanide solution, a small percentage segmented and a few even developed to the blastula stage, indicating that some effects of the original alkali treatment still remained and that reversal was not quite complete.

III. Fate of Eggs Which Did Not Receive the Cyanide Treatment.

The rates of oxidations in eggs which had received the acid or alkali treatment and had not been transferred to the cyanide solution but kept instead, until next day, in normal sea water, were also determined and the results are shown in Table IV.

TABLE IV.

No. of experiment	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Oxygen consumed, c. mm. per hr.													
Untreated eggs	30	30	18	28	20	24	10	17	19	18	24	11	16	9
Treated eggs (acid or alkali treatment) about 1–2 hours after treatment	62	53	50	49	27	80	17	56	26	28	37	49	48	31
Treated eggs next day	39	58	62	31	34	38	20	15	52	80	93	23	53	19

It will be noted that the figures for the oxidations on the second day are somewhat inconsistent. In some experiments the rate of oxidations has fallen to a figure not much greater than the original rate, while in other experiments it remains as high as or even higher than the increased rate due to membrane formation by acid or alkali treatment.

There is also considerable variation exhibited in the magnitude of the response to the acid or alkali treatment; in some experiments an increase in oxidations of about 400 per cent is noted, while in some the increase is less than 100 per cent.

The explanation for some of these apparent inconsistencies is fairly simple and lies in the fact that the magnitude of the response of sea urchin eggs to a given treatment by acid or alkali is not always the same, the variations being due to differences in sensitivity in the eggs of individual females and perhaps also to differences in room temperature during the several experiments. This accounts for the varying percentage increase in the rate of oxidations on a given treatment with acid or alkali and also for an unequal resistance to the disintegrating effects of such treatment, observed in the eggs of different females. It also accounts for differences observed in the rate of oxidations in untreated eggs preserved for some time in sea water in the laboratory. These frequently show a considerable spontaneous increase in a relatively short time, as will be seen from the figures given in Table V.

TABLE V.

No. of experiment	8	9	10	11	12	13	14	15	16
				Oxygen consumed, c. mm. per hr.					
Untreated eggs	24	10	17	19	18	24	11	16	9
Untreated eggs kept over night in normal sea water	29	17	17	67	36	80	23	26	16

In all these experiments except No. 10 a considerable increase has taken place. The magnitude of this increase, however, shows considerable variation.

IV. The Spontaneous Increase in Oxidations in Unfertilized Eggs.

In order to account more fully for the apparent inconsistency of the results in Tables IV and V, it is necessary to consider in some detail the spontaneous increase in oxidations in the eggs on standing. An endeavor was made to ascertain what factor is responsible for the phenomenon.

Loeb[5] showed that some of the eggs of certain females of the sea urchin *Strongylocentrotus purpuratus* formed fine gelatinous membranes spontaneously, when allowed to remain for some time, about 24 to 48 hours, in sea water at relatively low temperature. He found that these eggs sometimes segment, and if kept at a low temperature may even reach the sixteen cell stage. Loeb also found that if eggs which form membranes spontaneously are treated with hypertonic sea water, as in the usual method of artificial parthenogenesis, some will develop to larvæ.

In the eggs of *Arbacia* such spontaneous membrane formation is hardly ever observed. This may be due to the fact that in *Arbacia* eggs the membrane lies much more closely on the surface of the egg than is the case in *Strongylocentrotus*, and is therefore more difficult to observe. It is certainly true of the fertilization membrane and even more true of the membrane formed after the butyric acid treatment. A spontaneous segmentation is sometimes noted in *Arbacia*

[5] Loeb, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvi, 636.

eggs, but this never goes beyond the two or three cell stage. It is possible, therefore, that spontaneous membrane formation or some spontaneous alteration of the cortical layer also occurs, in the eggs of some females, in *Arbacia*.

Artificial membrane formation in *Strongylocentrotus* is always accompanied by a considerable increase in oxidations, whether the membranes have been called forth by butyric acid or by cytolytic agents such as saponin.[6] While the point has not been tested, it is reasonable to suppose that spontaneous membrane formation is also accompanied by a considerable increase in oxidations.

Loeb suggested that the spontaneous membrane formation in *Strongylocentrotus purpuratus* might be caused by the alkalinity of the sea water and it was thought desirable to ascertain whether the spontaneous increase in oxidations in *Arbacia* eggs takes place more readily in a slightly alkaline than in a slightly acid sea water. To this end the following simple experiment was carried out. Eggs in equal quantities were placed in a number of finger bowls. One-half of these bowls contained an artificial sea water made by mixing 100 cc. 0.5 M NaCl, 2.2 cc. 0.5 M KCl, 1.5 cc. 3/8 M $CaCl_2$, 7.5 cc. 3/8 M $MgCl_2$, and 3.5 cc. 3/8 M $MgSO_4$. This solution is acid to neutral red, while the normal sea water is slightly alkaline. All the various lots of eggs received an equal amount of washing. At intervals the rates of oxidations in eggs taken from the normal and from the artificial sea water were compared, the determination in both cases being performed in normal sea water. For this purpose the eggs from the artificial sea water were washed in normal sea water immediately before making the determination. The results are contained in Table VI.

[6] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 469.

TABLE VI.

Length of time eggs had remained in solution.	Normal sea water (slightly alkaline).		Artificial sea water (slightly acid).	
	Oxygen consumed per hr.	Condition of eggs.	Oxygen consumed per hr.	Condition of eggs.
hrs.	*c. mm.*		*c. mm.*	
3	12	Normal.	10	Normal.
20	19	Majority normal, some disintegrated.	10	"
26	19	Majority normal, some disintegrated.	13	"
44	57	Agglutinated and disintegrated.	27	Majority normal, some cytolyzed.
1½	11	Normal.	9	Normal.
19	11	"	10	"
44	73	All disintegrating.	19	Majority normal, some disintegrating.
1	10		9	
5½	19		18	
8½	17		16	
24	34	Many disintegrating.	37	
47	49	All "	29	Majority normal.
19	8		9	
29	31	Some disintegrating.	16	Normal.

These results, while incomplete, indicate that alkalinity plays an important rôle in the spontaneous increase in oxidations in the sea urchin egg, and though more experiments are required to determine its degree of responsibility, it is evident that the spontaneous disintegration of the eggs in sea water can be strongly inhibited by reducing the alkalinity.

The rôle played by the alkalinity of the sea water is also strikingly illustrated in experiments with butyric aid treatment of the eggs. In these experiments the butyric acid solution was made with artificial sea water possessing a faintly acid reaction instead of ordinary sea water which is faintly alkaline. The eggs were transferred from the butyric acid solution directly to artificial sea water or to normal sea water as the case might be, and the relative increase in the rate of

oxidations was determined 1 hour later. The results are given in Table VII.

TABLE VII.

Treatment.	2½ min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	5 min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	5 min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water	3 min. in 2 cc. 0.1 N butyric acid to 50 cc. artificial sea water.
Untreated eggs................	21.5	11	7	11
Treated eggs transferred to normal sea water............	26.0	18	16	46
Treated eggs transferred to artificial sea water............	16.0	9	6	11

When the eggs are transferred from the butyric acid solution to the faintly acid artificial sea water, the membranes are not formed so well as when the eggs are transferred to the slightly alkaline sea water. There is also some loss in the treatment with butyric acid in artificial sea water as the eggs in such a solution have a strong tendency to stick to the sides of the vessel. This accounts for the decrease in the rate of oxidations shown by the treated eggs when transferred to artificial sea water.

In the slightly acid sea water no increase in the rate of oxidations takes place following butyric acid treatment. The increase on transference to normal sea water shows, however, that the butyric acid treatment was sufficient for the purpose. The presence of excess of hydroxyl ions seems, therefore, to be necessary for the activation of the processes resulting in increased oxidations.

This result was forecast by Loeb[7] who found that eggs of *Strongylocentrotus purpuratus* when treated with butyric acid dissolved in neutral 0.5 M Ringer solution and transferred to neutral 0.5 M Ringer *per se*, formed only abnormal membranes and neither disintegrated nor developed. He suggested that the butyric acid treatment had not brought about the usual increase in oxidations but was inclined to attribute the abnormal behavior to causes other than hydroxyl ion concentration.

[7] Loeb, *Biol. Bull.*, 1915, xxix, 103.

One other possibility suggested itself in connection with the spontaneous increase in oxidations. It was observed that, when a considerable quantity of eggs remains in contact with only a small amount of sea water, a few of the eggs occasionally form membranes. It was also noticed that under such conditions the eggs appeared to disintegrate earlier than eggs which are less closely packed. This might be due to the presence, in considerable amount, in the surrounding sea water, of substances exuded by the eggs, which induced that critical change in the cortical layer of the egg responsible for membrane formation. Such eggs should exhibit an increased rate of oxidation.

In order to test this point, a suspension of eggs was equally divided. One portion was placed in a small amount of sea water under conditions of minimal evaporation while the other equal portion was placed in a relatively much larger quantity of sea water which was frequently renewed. Determinations of the rate of oxidations were then made at intervals, with the result given in Table VIII.

TABLE VIII.

No. of experiment	1			2			
No. of hours under given condition	4	23	26	4½	9	22	27¼
	Oxygen consumed, c. mm. per hr.						
Eggs kept in a small quantity of sea water	23	73	71	9	10	21	32
Eggs kept in a large quantity of sea water which was frequently renewed	22	38	52	10	24	25	48

It is evident from these figures that the spontaneous increase in the rate of oxidations continues in spite of dilution and removal of the egg exudates and is not even markedly inhibited thereby.

As a further control on the behavior of eggs on standing in sea water, a few determinations were made of the rate of oxidations in normal untreated eggs which had been kept over night in the sea water and sodium cyanide mixture. It is hardly necessary to state that the eggs were well washed in sea water and allowed to stand for

some time after removal from the cyanide solution before proceeding with the determination. These results are given in Table IX.

TABLE IX.

No. of experiment	14	15	16
	Oxygen consumed, c. mm. per hr.		
Untreated eggs	11	16	9
Untreated eggs on following day	23	26	16
Untreated eggs kept over night in NaCN solution	12	19	11

The changes responsible for the spontaneous increase in oxidations in unfertilized eggs on standing are evidently inhibited by preserving them in the sodium cyanide solution. This agrees with the old observation of Loeb and Lewis[8] that in potassium cyanide the life of unfertilized sea urchin eggs is prolonged.

On subjecting these eggs which had remained over night in the cyanide solution to treatment for 20 minutes with 0.3 cc. 0.1 N ammonium hydroxide in 50 cc. sea water, the oxidations were increased to an amount approximating that reached by eggs from the same lot when treated on the previous day, thus proving that the cyanide treatment had kept the eggs intact. This is shown by the results given in Table X.

TABLE X.

No. of experiment	14	15	16
	Oxygen consumed, c. mm. per hr.		
Untreated eggs	11	16	9
Eggs after NH₄OH treatment	49	48	31
Untreated eggs kept over night in NaCN solution	12	19	11
Eggs kept over night in NaCN solution and then treated with NH₄OH	34	42	39

In some of the experiments described, it was thought desirable to follow further the fate of eggs which had received the butyric acid

[8] Loeb, J., and Lewis, W. H., *Am. J. Physiol.*, 1901–02, vi, 305.

treatment. Accordingly, the treated eggs were preserved until the following day and the rate of oxidations was once more determined. A comparison of the figures obtained for treated eggs with those for untreated eggs kept during a similar period is of interest, especially in connection with the question of spontaneous oxidations. These·results are given in Table XI.

TABLE XI.

No. of experiment.	Time after treatment.	Untreated.	Treated.	Condition of treated eggs on second day.
		Oxygen consumed per hr.		
		c. mm.	*c. mm.*	
8	1 hr...................	24	80	
	Next day..............	29	38	All disintegrated.
9	1 hr...................	10	17	
	Next day..............	17	20	" "
10	1 hr...................	17	56	
	Next day..............	17	15	" "
11	1 hr...................	19	26	
	Next day..............	67	52	Some intact.
12	1 hr...................	18	28	
	Next day..............	36	80	Majority intact.
13	1 hr...................	24	37	
	Next day..............	80	93	70 per cent intact.
14	1 hr...................	11	49	
	Next day..............	23	23	Majority disintegrated.
15	1 hr...................	16	48	
	Next day..............	26	53	" "
16	1 hr...................	9	31	
	Next day..............	16	19	" " `

The untreated eggs were nearly ·always intact on the second day. It will be noticed that in some experiments the treated eggs show a diminution in oxidations on further standing, while in other experiments an increase occurs.

These results, at first glance somewhat anomalous, may be explained as follows: If the idea that the spontaneous increase in oxidations is due to the changes in the cortical layer which underlie membrane formation, is correct, then it is also probable that such alterations in the cortical layer of the egg will continue, influenced by the excess of OH ions in the sea water, even after membrane formation has been induced by acid treatment. In such a case the rate of oxidations will also continue to increase. Thus, in Experiments 11 and 13, the oxidations were not greatly raised by the butyric acid treatment. It happened, however, that in the untreated eggs the oxidations increased spontaneously to a high figure; consequently, in the treated eggs, the oxidations continued to increase, also spontaneously, until a correspondingly high figure was attained. In the other experiments, with the exception of No. 12, the oxidations increased considerably on treatment. Such eggs show no or only very slight further increase in oxidations on the following day, the maximum having already been reached as a result of the acid treatment. Generally, owing probably to disintegration, the oxidations have again diminished on the next day, by an amount roughly proportional to the degree of disintegration.

Another explanation is also possible though perhaps less probable, and is as follows: Warburg and Meyerhof[9] found that the oxidations in unfertilized sea urchin eggs are only slightly less after disintegration than before. Warburg[10] found that when fertilized eggs are caused to disintegrate, the rate of oxidations returns to the value for an equal quantity of unfertilized eggs. He concluded that the increase in oxidations on fertilization is bound up in the existence of the fertilization membrane. This idea resembles somewhat the *Hauptathmung* and *akzessorische Athmung* of Battelli and Stern.[11] Warburg[12] showed that the membrane called forth by the butyric acid treatment acts like the fertilization membrane in increasing the rate of oxidations. Assuming that on disintegration the rate of oxidations in the treated eggs similarly falls again to the rate in the unfertilized eggs, which

[9] Warburg, O., and Meyerhof, O., *Arch. ges. Physiol.*, 1912, cxlviii, 295.

[10] Warburg, O., *Arch. ges. Physiol.*, 1914, clviii, 189.

[11] Battelli, F., and Stern, L., *Biochem. Z.*, 1914, lxvii, 443.

[12] Warburg, *Z. physiol. Chem.*, 1910, lxvi, 305.

is probable, and assuming that the spontaneous increase in oxidations in unfertilized eggs is not caused by spontaneous membrane formation but by the action of the OH ions of the sea water independently of membrane formation, the results in Table XI can be readily explained.

In Experiments 12 and 13 the oxidations are slightly raised by the acid treatment and the spontaneous increase in the untreated eggs next day is considerable. A spontaneous increase has similarly occurred in the treated eggs, and as the eggs had not disintegrated the figures remained high. In the remaining experiments in which the treated eggs had disintegrated on the second day, the increased rate of oxidations has diminished again to a value corresponding to that for the untreated eggs. Where no spontaneous increase has taken place, the increased rate due to treatment has dropped to the original value. In those cases where some spontaneous increase took place the final value on the second day for both treated and untreated eggs agrees closely.

V. What Causes the Reversal Effects?

It has been shown that after the reversal of the action of butyric acid as demonstrated by Loeb, the rate of oxidations is the same as the rate existing before treatment, and the several questions subsidiary to the main issue have also been answered fairly satisfactorily.

The next question for consideration would appear to be, how does the sodium cyanide solution accomplish the reversion? We know that it suppresses oxidations and development, but there is no *a priori* reason why a temporary suspension of induced cell activities should result in a reversion to the reactions of an unfertilized egg. It is to be remembered, in this connection, that, as Loeb showed, chloral hydrate acts similarly to sodium cyanide in causing a reversion, and the chloral hydrate compared to sodium cyanide has a relatively slight effect in depressing the oxidations in sea urchin eggs. The following experiments carried out with *Arbacia* eggs serve to illustrate this.

TABLE XII.

	Relative oxygen consumption.
Fertilized eggs in normal sea water......................	1.00
" " " 50 cc. " " + 0.22 cc. 0.1 per cent NaCN.....	0.15
" " " 50 " " " " 0.22 " 0.1 " " "	0.27
" " " 50 " " " " 0.15 " 0.1 " " "	0.20
" " " 40 " " " " 10.00 " 0.5 " " chloral hydrate..	0.67
Fertilized eggs in 42.5 cc. sea water + 7.5 cc. 0.45 per cent chloral hydrate	0.61
" " " 44.0 " " " + 6.0 " 0.5 " " " "	0.73

These experiments were made in the course of another investigation and the concentrations are not those most suitable for the reversion of the effects induced by butyric acid. The concentrations used in the reversal experiment are 0.4 cc. 0.1 per cent sodium cyanide or 5 cc. 0.5 per cent chloral hydrate per 50 cc. sea water mixture, consequently the depression of oxidations during the reversal process is probably less for chloral hydrate and greater for sodium cyanide than is shown by the above figures.

It is probable, therefore, that the main factor concerned in bringing about reversion is the suppression of the developmental processes, the lowering of the rate of oxidations being merely the means of suppressing cell division.

In conclusion, the writer wishes to express his thanks to Professor Jacques Loeb for advice and criticism.

SUMMARY.

1. The rate of oxidations was determined in sea urchin eggs in which development had been initiated and later reversed by the methods of Loeb.

2. It was found that the rate of oxidations, which increases after initiation of development, returns, after reversal, to approximately the original rate of the untreated eggs.

3. It was found that on retreating the reversed eggs so as to initiate development once more the oxidations are again increased.

4. Eggs in which initiated development had been reversed could be fertilized by sperm, and also in most cases could be caused to develop by the usual methods of artificial parthenogenesis.

5. The oxidations in eggs in which development had been initiated but not reversed were studied, as were also the oxidations in untreated eggs from the same batch.

6. It was observed that the rate of oxidations in untreated eggs rose spontaneously on standing, sometimes showing a 300 per cent increase.

7. The probable cause of the spontaneous increase was found in the alkalinity of the sea water.

8. It was shown that the oxidations do not increase in eggs treated with butyric acid when transferred to faintly acid sea water.

9. The cause of the reversal is discussed, and it is shown to lie mainly in the suppression of developmental processes.

ANTIBLASTIC IMMUNITY.

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As a result of the study of the natural or acquired resistance of animals against infection with living microorganisms, many previously unknown properties of the cells and fluids of the body have been discovered. The value of these properties as resistance factors is dependent upon the degree of antagonism which they manifest to invasion with living foreign elements. On the other hand, virulence of bacteria may be said to be proportional to the strength of defence which they possess against such antagonistic forces. In the face of such opposition, however, the bacterium must not only maintain its life, but in order to increase must be able to obtain a proper food supply. The mechanism by means of which bacteria extract from living tissue the substances essential to their nutrition is not well understood, nor can we say to what extent the animal body opposes this appropriation of its own supplies. It is in the hope of throwing light upon this obscure problem that the following study has been undertaken.

The microorganism used was the pneumococcus, not because it is especially suitable, but because many of the materials necessary for the experiments were easily obtainable. The hypothesis upon which our efforts are based is that bacteria do not assimilate all of their foodstuffs in the condition in which they exist in the medium of their environment, but that certain changes must be effected before absorption occurs. The preparation of nutritional substances may take place upon the surface of the bacterial cell or in its immediate neighborhood, and this function, when carried on within the substance of a living animal, may be opposed by certain inhibitory forces. The interaction of these phenomena may play an important part in resistance and immunity to infection.

By immunization of the horse to pneumococcus, a serum can be prepared which protects susceptible animals against many times the fatal dose of virulent pneumococci. A complete understanding of the mode of action of this serum is still lacking. However, the fact has been observed and confirmed that antipneumococcus serum possesses neither bactericidal nor bacteriolytic action *in vitro*. Indeed, pneumococcus is known to grow in considerable concentration of its homologous immune serum. In the following experiment (Table I) is shown the rate of growth of pneumoccoccus in homologous and in heterologous antipneumococcus serum and in normal horse serum The amount of growth was determined by the plate method.

TABLE I.

Experiment I.   Inhibition of Growth of Pneumococcus by Antipneumococcus Serum.

Serum 0.1 cc.	Culture 0.00001 cc.	Immediately.	After 3 hrs.
Antipn. serum Type I...........	Pn. I	230 colonies.	216 colonies.
" " " II...........	" I	200 "	630 "
Normal.......................	" I	200 "	1,400 "

This experiment shows clearly that a marked inhibition of the growth of pneumococcus occurs in antipneumococcus serum, as compared with normal horse serum. The inhibitory effect of homologous immune serum is greater than that of heterologous immune serum; in fact, in the former serum no increase in the number of pneumococci has taken place in three hours. The inhibition of growth may be explained in four ways: first, that agglutination occurs in immune serum, and that the failure to increase is apparent only; second, that the formation of threads is responsible for the apparent inhibition; third, that we are dealing with the bacterial lag of freshly planted cultures; and fourth, that active interference with the growth phenomena of the organisms has occurred. That the first two reasons are entirely responsible for the inhibition of growth is disproven by the fact that a marked delay in development occurs in heterologous immune serum in which no agglutination whatever takes place and in which thread formation is not more extensive than in normal serum. The latent period that marks the growth of freshly planted

cultures of bacteria cannot explain the inhibition, inasmuch as this would affect the culture in normal serum as well as that in immune serum, since the same culture of pneumococcus was used for seeding and the conditions of cultivation were similar in each tube. We are forced, then, to conclude that these various phenomena do not entirely explain the inhibition of growth, and that such inhibition as occurs is largely dependent upon some property of immune serum which adversely affects the circumstances of multiplication. The inhibitory influence of immune serum is manifest only for a relatively short period of time, for if plates are made at the end of twenty-four hours, the pneumococcus is found to have overcome the inhibition, and innumerable colonies develop from comparable amounts of all the cultures.

Carbohydrate and protein form the main food supply of most bacteria. In our hypothesis we have suggested that these substances may not be absorbed unchanged and that some form of preparation may occur at the surface of the bacterial cell. If immune serum is added to the medium in which the cell is growing, the changes necessary for the development of food substances appropriate for assimilation would be subject to the influence of any inhibitory bodies present in the immune serum. In the following experiments evidence is brought that antipneumococcus serum possesses the power to inhibit both the splitting of protein and the fermentation of sugar by pneumococcus. The organisms were grown in serum broth for twenty-four hours. The amount of protein splitting has been estimated by the increase in amino nitrogen determined by the method of Van Slyke.

The following experiment is one of a series showing the degree of protein splitting that occurs when pneumococci are grown in broth containing normal horse serum, and the effect of substituting antipneumococcus serum for normal serum in the mixtures. The estimations of the increase in amino nitrogen were made after twenty-four hours' incubation at 37° C., a period at which marked growth of the pneumococcus had occurred both in the tubes containing normal serum, and in those containing immune serum. In those tubes in which the pneumococcus has grown in its homologous serum, agglutination has occurred. There has been, however, no agglutination either in normal serum or in heterologous immune serum.

TABLE II.

Experiment II. Inhibition of Digestion of Protein by Pneumococcus with Anti-pneumococcus Serum.

	Increase in amino nitrogen per cc.
Pn. Type I + 1 cc. normal horse serum + 4 cc. broth..........	0.21 cc. nitrogen.
" " I " 1 " antipn. serum Type I + 4 cc. broth.......	0.02 " "
" " I " 1 " " " " II " 4 " "	0.06 " "
" " II " 1 " normal horse serum " 4 " "	0.21 " "
" " II " 1 " antipn. serum Type I " 4 " "	0.14 " "
" " II " 1 " " " " II " 4 " "	0.06 " "

The figures of this experiment (Table II) make it strikingly evident that the addition of antipneumococcus serum to growing cultures of pneumococcus markedly diminishes, in some instances almost to the point of extinction, the production of amino-acids by the organism. The formation of amino-acids in mixtures of broth and normal horse serum has been found generally equivalent to the amount found in broth alone. Certain other normal animal sera have, however, been observed which inhibit the process in varying degrees. Inhibition by homologous immune serum is most complete, although, as in the experiment showing inhibition of growth of pneumococcus, heterologous immune serum possesses considerable inhibitory power. Whether the increase in amino-acid is due to endogenous or exogenous metabolism cannot, of course, be definitely determined. We believe, however, that, in the utilization of protein, the pneumococcus effects a splitting of the protein before absorption, and that the increase in amino-acid represents the excess of protein split, over that used up in the process of growth.

Carbohydrate, as is well known, is an important part of the food supply of most bacteria, and the great variety of sugars that many organisms ferment is extraordinary. That such a splitting of sugars is in some way associated with the nutrition of microorganisms seems a logical assumption. We have found that the addition of antipneumococcus serum to cultures of pneumococcus containing such sugars as glucose, saccharose, lactose, and inulin inhibits in varying degrees the fermentation of the sugars by the organism. Inhibition of fermentation of inulin is most complete, and an example of this inhibition is given in Experiment III (Table III). Pneumococcus, when added to serum water media containing inulin, and litmus as

an indicator, ferments the inulin which results in acidification of the medium and coagulation of the serum.

TABLE III.

Experiment III.

	24 hrs.	48 hrs.	72 hrs.	5 days.
Pn. Type I + inulin..........................	++	++	++	++
" " I " antipn. serum Type I + inulin.	−	−	−	−
" " I " " " " II " "	Sl. ac.	Ac.	++	++

+ + indicates complete acidification and coagulation; + indicates acid and incomplete coagulation; ± indicates acid and beginning coagulation; V. sl. ac. indicates very slight acidification; Sl. ac. indicates slight acidification; Ac. indicates slight acidification and no coagulation; − indicates no acidification or coagulation.

Experiment III shows that the addition of homologous immune serum to a culture of pneumococcus in inulin completely suspends fermentation of the inulin. Heterologous immune serum delays the reaction, but does not entirely inhibit it. Fermentation of the sugars more actively attacked by pneumococcus, such as glucose, lactose, and saccharose, is not inhibited to the same degree as that of inulin. Determination of the rate of production of acid in cultures containing such easily fermentable sugars shows that, in the early hours of growth, the formation of acid is markedly delayed by the presence of immune serum. After twenty-four hours, however, the acid concentration may reach the same degree in all tubes and, in general, represents the grade of acidity at which pneumococcus ceases to grow. The splitting of carbohydrates by bacteria probably occurs at the surface of the bacterial cell, as is thought to be the case in fermentation of sugar by yeast, and the anti-enzymotic forces of immune serum in all probability exert their antagonistic action at this point.

A study of human blood serum obtained at intervals during the course of an attack of lobar pneumonia shows that bodies having an anti-enzymotic action similar to that of immune serum develop during the period of recovery from the disease. The tests were made in the same manner as those in which an artificially prepared immune

serum was used. The two following experiments are typical of the
results obtained (Tables IV and V).

TABLE IV.

*Experiment IV. Inhibition of Digestion of Protein by Pneumococcus with Human
Serum in Lobar Pneumonia. Infection with Pneumococcus Type II.*

	Increase in amino nitrogen per cc.
Pn. Type II + 2 cc. broth + 0.5 cc. serum, 5 days before crisis.....	0.16 cc. nitrogen.
" " II " 2 " " " 0.5 " " at crisis...............	0.02 " "
" " II " 2 " " " 0.5 " " 9 days after crisis......	0.02 " "

TABLE V.

*Experiment V. Inhibition of Fermentation of Inulin by Pneumococcus with Human
Serum in Lobar Pneumonia. Infection with Pneumococcus Type I.*

	24 hrs.	48 hrs.	72 hrs.	5 days.
Inulin + Pn. Type I..............................	++	++	++	++
" " " " I + serum 8 days before crisis...	±	++	++	++
" " " " I " " 6 " " " ...	+	++	++	++
" " " " I " " just after crisis.......	—	V.sl.ac.	Sl.ac.	Ac.
" " " " I " " 7 days " "	Sl.ac.	Ac.	±	±

These experiments show clearly that at the crisis of lobar pneu-
monia substances appear in the serum either for the first time, or in
greatly increased amount, which have the power of inhibiting the
proteolytic and glycolytic activities of the pneumococcus.   The period
of development of these substances corresponds in time with that
of other immune bodies which have been recognized in the serum
of individuals with lobar pneumonia.

DISCUSSION.

The series of experiments presented in this paper demonstrate the
following facts. Antipneumococcus serum possesses the power of
inhibiting for a certain period of time the multiplication of pneu-
mococci.   In conjunction with this capacity, it has also the power
of inhibiting in varying degree the proteolytic and glycolytic functions
of pneumococci. This power is present to a limited extent in the

sera of certain normal animals and absent in others, and in human sera during the course of an attack of lobar pneumonia it appears for the first time or increases markedly at the critical period of the disease. From these facts we are led to assume that retardation of growth is, in part at least, dependent upon inhibition of metabolic function. The observation that immune serum possesses in high degree the powers described, suggests that these properties play an important part in resistance and immunity to infection with pneumococcus. Investigators have demonstrated previously that certain other immune sera possess analogous qualities; such as the inhibition of pigment production by *Bacillus pyocyaneus* (1), the liquefaction of gelatin by *Staphylococcus pyogenes aureus* (2), and the formation of methemoglobin by pneumococcus (3). We have chosen the term "antiblastic immunity" as descriptive of this phenomenon, in order to indicate that the forces at work are antagonistic to the growth activities of the organism. Ascoli (4) coined the term several years ago (βλαστειν, to grow). From his studies in anthrax immunity, he was led to suppose that the latter was in part dependent upon the inhibition of formation by *Bacillus anthracis* of a capsule which is a prerequisite for its successful development in the animal body, and he ascribed to anti-anthrax serum an antiblastic action, directed against the metabolic activity of this organism. A concrete interpretation of this phenomenon as applied to the growth of pneumococcus and the inhibitory influence of immune serum is as follows: Pneumococcus, in order to grow, must obtain a sufficient supply of protein and carbohydrate; these substances are furnished by the environmental medium, but probably require, to render them suitable for absorption, preliminary preparation in the nature of digestion. This change is effected at the surface of the bacterial cell and the integrity of this digestive zone is essential to the growth of the bacterium. Anti-enzymotic bodies such as have been demonstrated in immune serum act at the point of contact of the cell with its environment, and influence in an unfavorable manner the nutritional processes there carried on, and the consequence of such action is retardation or inhibition of growth. It is possible that capsule formation represents on the part of the organism an attempt to protect the function of the digestive zone. Should the foregoing prove

to be a correct explanation of the phenomenon observed, considerable light would be thrown on the obscure mechanism by means of which parasitic bacteria establish themselves in animal tissues, and on the forces mobilized by the animal body in opposition to such invasion.

CONCLUSIONS.

1. Antipneumococcus serum possesses the power of inhibiting for a certain period of time the multiplication of pneumococci.

2. It also has the capacity of inhibiting the proteolytic and glycolyt'c functions of pneumococci.

3. This power is acquired for the first time or appears in increased amounts in human serum at the time of crisis in lobar pneumonia.

4. The retardation of bacterial growth is thought to be dependent upon the inhibition of metabolic function due to the presence of anti-enzymotic substances in antipneumococcus serum. To this phenomenon we have applied the term antiblastic immunity.

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1. Ghéorghiewsky, *Ann. de l' Inst. Pasteur*, 1899, xiii, 298.
2. von Dungern, *Centralbl. f. Bakteriol.*, *1te Abt., Orig.*, 1898, xxiv, 710.
3. Cole, R., personal communication.
4. Ascoli, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1908, ҳlvi, 178.

THE DETERMINATION OF UREA BY THE UREASE METHOD.

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In a recent number of this *Journal* Fiske[1] recommends as an improvement to the accuracy of our technique for urea determination[2] the use of a slower rate of aeration in driving the ammonia over into the standard acid, and the consequent prolongation of the time for aeration to an hour, in order to insure complete absorption of the ammonia by the acid. This modification and the consequent loss of time, however, are unnecessary if the directions given in our paper are followed; the accuracy attainable is limited only by that of the measurements and standard solutions. The conditions necessary for both complete and rapid absorption were studied rather exhaustively in experiments which it did not seem, at the time, essential to publish, but it now appears that certain of the principles on which our procedure was based must be stated more clearly.

1. One of the important factors in complete absorption is the height of the absorbing column of standard acid. We utilized as containers of the absorbing solution test-tubes of 100 cc. capacity and the usual shape (about 25 x 200 mm.). In such tubes 25 cc. of acid make a column 50 to 60 mm. high. Instead of these Fiske used "bottles," in which the column of acid is presumably much shorter. This change is the most obvious reason for the longer period of aeration required.

2. Another point which may bear expansion is the reason for our

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[1] Fiske, C. H., *Jour. Biol. Chem.*, 1915, xxiii, 455.

[2] Van Slyke, D.D., and Cullen, G. E., A Permanent Preparation of Urease, and Its Use in the Determination of Urea, *Jour. Biol. Chem.*, 1914, xix, 211.

statement (page 221): "If one uses *a moderate current of air for the first minute* every particle of ammonia is absorbed."

The necessity for caution at the start is that, with a given air current, the rate at which ammonia is carried over at the beginning of the aeration is enormously faster than afterwards. Consequently what caution is required should be concentrated on retarding the first burst of ammonia evolution. After that is over, it is almost impossible, with the apparatus and conditions described in our paper, to drive the ammonia over into the acid so fast that any of it escapes absorption. It does no harm, of course, to prolong the period of relatively slow aeration, but it is unnecessary. It is essential only that the fact be kept in mind that the first stage of aeration is the one that requires all the caution in regulating the air current. After this period is past, it is only a waste of time not to use as fast a current as is possible without loss of liquid from spattering. A much faster current can be used with a drop of caprylic alcohol to prevent foaming than with any other practicable agent, such as kerosene, for this purpose with which we are acquainted. We usually employ an air current of about 5 liters per minute, which insures complete aeration of the ammonia in 15 minutes.

Experiment 1.—The relatively high rate at which ammonia is evolved at the beginning of the aeration, and the constantly decreasing rate during the later stages, are indicated by the following experiment. 5 cc. portions of 0.0991 N ammonium sulfate (5 cc. = 24.78 cc. 0.02 N ammonia) were measured into each of a series of tubes arranged for aerating as illustrated on page 217 of our former paper, with the addition that a second absorption tube, containing 1 cc. of 0.01 N acid plus 10 cc. of water, was inserted after each pair of tubes. 5 cc. portions of 1:1 potassium carbonate solution were added, and the aeration was conducted with an air current of 5.3 liters per minute from the start. The ammonia was absorbed into 30 cc. portions of 0.02 N acid. At different intervals the successive sets of absorption tubes were disconnected and the ammonia which had been driven over was titrated. The results are given in Table I.

The air current was determined by Kober's[3] scheme of measuring the time required to draw 10 liters of water from one bottle into another. The water levels in both were so arranged that positive water pressure during the first half of the transfer was balanced by negative during the second; and the connecting tube was so large (1.5 cm. inner diameter) that the flow of water was not appreciably retarded.

[3] Kober, P. A., and Graves, S. S., *Jour. Am. Chem. Soc.*, 1913, xxxv, 1594.

The analyst will find it worth while to standardize in this manner the air current and calculate the length of time required to drive 100 liters of air through the apparatus; as this amount with the apparatus described in our paper appears sufficient, with a good margin of safety, to drive over all the ammonia.

TABLE I.

Time of aeration.	Volume of air passed.	0.02 N HCl neutralized in first receiving tube.	0.02 N HCl neutralized in second receiving tube.	Total HCl neutralized.
min.	*liters*	*cc.*	*cc.*	*cc.*
1	5.3	13.5	0.08	13.58
2	10.6	20.3	0.08	20.38
3	15.9	22.8	0.08	22.88
5	26.5	24.1	0.08	24.18
10	53.0	24.6	0.09	24.69
15	79.5	24.7	0.09	24.79
25	132.5	24.7	0.08	24.78

At the beginning of the aeration the speed of evolution was so great that more than 55 per cent of the total ammonia was driven over in the first minute, 0.32 per cent being driven past the first receiving tube and caught in the second. During the second minute 27 per cent of the total ammonia was driven over. None passed the first tube during the second or following minutes. It is evident, therefore, that if the rate of evolution during the first 2 minutes had been reduced by cutting down the air current to one-half, no ammonia at all would have been lost. As it was, with a full air current from the start, the loss was only 0.3 per cent of the total, 99.7 per cent being retained in the first receiving tube. The amount of ammonia aerated was equal to the maximum ordinarily obtained in urine analyses.

It is a point of interest that, air current and other conditions being constant, the rate at which ammonia is driven over is exactly proportional to the amount present. The rate of evolution follows the same law $\frac{dx}{dt} = k\,(a - x)$ as the rate of a monomolecular reaction.

Thus, taking a (total amount of ammonia present) equivalent to

24.78 cc. of 0.1 N acid, x being the amount driven over after t minutes, we have:

TABLE II.

t	x	$k = \frac{1}{t} \log_e \frac{a}{a-x}$	$k (a - x)$ Calculated rate of aeration after t minutes.
	Cc. 0.02 N NH₃		Cc. 0.02 N NH₃ per min.
min.			
0			20.2
1	13.58	0.794	9.0
2	20.38	0.867	3.6
3	22.88	0.857	1.5
5	24.18	0.744	0.5

Average...............................0.82

The value, 0.82, of k indicates that at any given moment ammonia was being removed at the rate per minute of 82 per cent of the ammonia present.

The figures in the last column are calculated from the velocity equation $\frac{dx}{dt}$ = rate of aeration = $k\ (a - x) = 0.82\ (24.78 - x)$. They show that after 1 minute the rate of aeration had fallen parallel with the still remaining amount of ammonia, $a - x$, to less than half the initial rate, and that after 2 minutes the rate had fallen to one-sixth the initial velocity.

Experiment 2.—In order to ascertain the effect of using a half-speed air current during the first minute, four sets of tubes were aerated for 15 minutes in precisely the same manner as in the above experiment, except that during the first minute an air stream of 2.6 liters per minute was used, the 5.3 liter current being applied after 1 minute's aeration. The amounts of 0.01 N acid neutralized in the guard tubes were 0.04 to 0.05 cc., equivalent to 0.02 to 0.025 cc. of 0.02 N acid, or 0.08 to 0.10 per cent of the total ammonia, 99.90 to 99.92 per cent being absorbed by the 0.02 N acid in the first receiving tubes.

As an error not exceeding 1 part per 1,000 may be regarded as negligible for most purposes, we believe that no alteration need be made in the directions in our first paper for the technique of aeration. If the analyst wishes, however, to make sure that even less than 1

part of ammonia per 1,000 escapes absorption, he may continue the half-current for 2 minutes instead of 1.

3. Since our former publication we have found, like Fiske, that addition of potassium carbonate in approximately saturated solutions, to set free ammonia for aeration, is preferable to its addition in solid form, and have privately recommended this modification. The difference is one of convenience rather than accuracy. Contrary to Fiske's assumption, the increase in volume of liquid from 5 cc. to 10 cc. does not significantly retard the evolution of ammonia during aeration. As we stated (page 221) after systematic tests, "The volume of the solution (in a 100 cc. test-tube) from which ammonia is being removed can be varied from 5 cc. to 25 cc. without appreciably altering the time required to drive off the ammonia," as long as the solution contains potassium carbonate in a concentration of at least 1 gm. to 2 cc. of solution.

4. Although, as we showed and Fiske repeats, urease in water extracts containing primary and secondary phosphate preserves most of its activity for several weeks when kept at 0°, ammonia is likely to form in such extracts, even at 0°, and in sufficient amounts to cause relatively large errors, particularly in blood analyses. The loss of activity also is rather insidious, for the reason that an extract which has remained for a long time almost without change loses activity at an unexplainably rapid rate after it has once begun to deteriorate. The safest way is to use a standardized preparation of dry urease, and prepare fresh solutions daily as required.

5. We have found it necessary, especially in blood analyses, to correct for ammonia in the reagents. The chief source of the correction is the potassium carbonate. Blank determinations are run on every lot of reagents, using 1 cc. of 10 per cent urease solution, 5 cc. of the 0.6 per cent phosphate solution, and 10 cc. of saturated potassium carbonate solution, and aerating into 0.01 N acid. The correction obtained has been as high as 0.12 cc. of 0.01 N acid, but with the carbonate now in use (Merck's U. S. P. viii) is 0.05 cc. of 0.01 N acid.

6. In closing we mention one essential precaution which we have always observed as a matter of course, but neglected to state in our original paper. As a result at least one colleague has had much un-

necessary trouble.   If a test-tube and stopper are used as container, first for the strong carbonate solution, then, during a subsequent aeration, for the 0.02 N or 0.01 N acid solution, the tube or stopper, no matter how thoroughly washed with distilled water, is likely to carry a trace of alkali into the highly dilute acid and appreciably affect results.   Therefore separate sets of tubes and stoppers must be employed as containers for the standard acid, and these must not be used for alkaline solutions.   In case a tube that has been used for an alkaline solution is employed for standard acid, both tube and stopper are washed first with dilute acid, then with water.

It may be well also to call attention to the fact that the titration value of 0.01 N or 0.02 N alkali in ordinary glass vessels increases perceptibly from week to week, because the solution gradually dissolves more alkali from the glass.   This action is particularly rapid in such a long narrow vessel as a burette.   Alkali of 0.01 N concentration may appreciably increase its titration value as the result of standing for even a day in a burette.

TREATMENT OF TYPHOID FEVER BY INTRAVENOUS INJECTIONS OF POLYVALENT SENSITIZED TYPHOID VACCINE SEDIMENT.

STUDIES IN TYPHOID IMMUNIZATION. VI.*

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Since the work of Fraenkel[1] in 1893, killed preparations of the typhoid bacillus have been injected subcutaneously as a means of treatment in typhoid fever. Little interest was at first awakened by the suggestive results of Fraenkel except in a discussion of the specificity of his treatment (Rumpf,[2] Kraus and Buswell,[3] Presser[4]). In 1902 Petruschy[5] used a combination of vaccine and immune serum in typhoid, and in 1908 Pescarolo and Quadrone[6] advocated the use of a

[1] Fraenkel, E.: Ueber spezifische Behandlung des Abdominaltyphus, Deutsch. med. Wchnschr., 1893, xix, 985.

[2] Rumpf, T.: Die Behandlung des Typhus abdominalis mit abgetodteten Cultures des bacillus Pyocyaneus, Deutsch. med. Wchnschr., 1893, xix, 987.

[3] Kraus, F., and Buswell, H. C.: Ueber die Behandlung des Typhus abdominalis mit abgetodteten Pyocyaneus Culturen, Wien. klin. Wchnschr., 1894, vii, 511, 595.

[4] Presser, L.: Ueber die Behandlung des`Typhus abdominalis mit Injektionen von Culturflüssigkeiten von Bac. Typhi und Bac. Pyocyaneus, Ztschr. f. Heilk., 1895, xvi, 113.

[5] Petruschy, J.: Spezifische Behandlung des abdominal Typhus, Deutsch. med. Wchnschr., 1902, xxviii, 212.

[6] Pescarolo, B., and Quadrone, C.: Aktive Immunisation durch subcutane Injektionen lebender Typhusbazillen bei Eberth'scher Infektion, Zentralbl. f. inn. Med., 1908, xxix, 989.

living, avirulent typhoid culture.   Following the interest in vaccine therapy awakened by Wright, increasingly frequent reports on the possible value of typhoid vaccines in typhoid fever have appeared. In 1912 Callison[7] summarized the results obtained by numerous authors, chiefly English and American, in 747 cases, and in 1915 Krumbhaar and Richardson[8] could collect over 1,800 cases reported on by forty authors.   It is known that many physicians, and probably an increasing number of them, use vaccines in typhoid because they find they do no harm and they believe them to do some good.   The best studied groups of cases treated in this manner, however, give ground for little unrestrained enthusiasm, and certainly no claim that any such type of specific therapy has been attained as is the case in diphtheria and epidemic meningitis.   The best that may be said is that the ordinary type of killed typhoid vaccine administered subcutáneously in controlled groups of cases may cause a shortening of the course of the disease, a lower mortality, and perhaps fewer relapses and complications.   We purposely refrain from further analysis of the ordinary type of vaccine therapy which has been described, since we are to deal with methods and results which we believe constitute a new era in the specific treatment of typhoid fever.

In the last few months reports on the intravenous injection of ordinary heat-killed vaccine (Thiroloix and Bardon,[9] Kraus and Mazza[10]) and particularly on the intravenous injection of sensitized vaccines (Ichikawa[11]) in typhoid fever, have led us to anticipate a far more hopeful future in combating this disease than has hitherto seemed likely.   Before proceeding to a consideration of the results recorded by several authors in actual cases, we may be pardoned for repeating

[7] Callison, J. G.:   The Therapeutic Use of Vaccines in Typhoid Fever, Am. Jour. Med. Sc., 1912, cxliv, 350.

[8] Krumbhaar and Richardson: The Value of Typhoid Vaccine in the Treatment of Typhoid Fever, Am. Jour. Med. Sc., 1915, cxlix, 406.

[9] Thiroloix and Bardon: Vaccin Typhique intraveineux, Centralbl. f. Bakt. Ref., 1914, lx, 212 (3d ref.).

[10] Kraus, R., and Mazza, S.:   Zur Frage der Vakzinetherapie des Typhus abdominalis, Deutsch. med. Wchnschr., 1914, xl, 1556.

[11] Ichikawa, S.:   Mitteil. d. med. Gesellsch., zu Osaka, April, 1912, x, No. 5; Abortive Treatment of Typhoid and Paratyphoid, Sei-I-Kwai Med. Jour., Tokyo, 1914, xxxiii, No. 12, p. 73, or Ztschr. f. Immunitätsforsch., 1914, xxiii, 32.

the experimental evidence by which we were led to a practical consideration of this problem before most of these observations were made.

In a previous article in this series Gay and Claypole[12] described a new and specific form of hyperleukocytosis which occurs in immunized rabbits on intravenous reinjection of the specific antigen (bacteria, red blood cells, serum). We found, for example, that the injection of living typhoid bacilli, or of typhoid vaccine in a typhoid immune rabbit, caused the leukocytes to fall in the first two or three hours and then to rise to critical levels that occurred at about the twelfth and again at the twenty-eighth hour. At the latter period leukocyte counts of 150,000 per cubic millimeter were not uncommon, a surprising rise from the normal count of from 8,000 to 12,000, and one that has not been attained, so far as we are aware, by other experimental method. These hyperleukocytic crises, moreover, are coincident with the destruction of the typhoid bacillus in the immune animal, and would appear to be the cause of it. The same dose of typhoid bacilli in a normal rabbit produces a distinct but markedly lower grade of hyperleukocytosis. We logically regarded the extreme grade of hyperleukocytosis as dependent on the presence of antibodies (tropins) in the immune animal, an hypothesis that we were able to verify by obtaining similar results in normal rabbits by the use of tropinized (sensitized) cultures of *B. typhosus;* that is to say, cultures that had been treated with an immune serum. From these experiments it seemed reasonable to express the opinion that the intravenous injection of sensitized typhoid vaccine offered a possibly successful method of treating typhoid fever.[13] As a preliminary to the intended application of the sensitized typhoid vaccine sediment, which we advocate for prophylactic use against typhoid fever,[14] in the treat-

[12] Gay, F. P., and Claypole, E. J.: Specific Hyperleukocytosis: Studies in Typhoid Immunization, IV, THE ARCHIVES INT. MED., 1914, xiv, 662.

[13] Gay, F. P., and Claypole, E. J.: Specific Hyperleukocytosis: Studies in Typhoid Immunization, IV, THE ARCHIVES INT. MED., 1914, xiv, 669.

[14] Gay, F. P., and Claypole, E. J.: An Experimental Study of Methods of Prophylactic Immunization Against Typhoid Fever: Studies in Typhoid Immunization, V, THE ARCHIVES INT. MED., 1914, xiv, 671.

ment of this disease, we tested its curative effect in rabbits that had been made "carriers" of the typhoid bacillus.[15] A few of these animals were apparently freed of the typhoid bacillus by the intravenous injection of the sensitized vaccine; that a majority of them remained unaffected is due to the inaccessibility of the microorganism in the gallbladder of such carrier rabbits. Further preliminary experiments have shown us that relatively large doses of sensitized typhoid vaccine can be given with safety directly into the circulation of rabbits and monkeys, and even when these animals have been partially immunized against *B. typhosus*. The latter condition owing to the presence of agglutinins, was aimed to simulate the condition in typhoid fever.[16]

On the basis of this experimental evidence accumulated in 1913 and 1914, we were prepared to attempt treatment of human cases, but no opportunity presented itself until early in 1915. Since February of this year we have been privileged to examine over 100 cases of clinically suspected typhoid, to carry out in most of them our own laboratory examinations, and to cooperate in the treatment of the great majority of them in which a diagnosis of typhoid fever could be fully confirmed. This study has been made possible by the hearty interest and cooperation of over fifty physicians in Berkeley, Oakland, San Francisco and their environs.[17]

[15] Gay, F. P., and Claypole, E. J.: The "Typhoid Carrier" State in Rabbits as a Method of Determining the Comparative Immunizing Value of Preparations of the Typhoid Bacillus: Studies in Typhoid Immunization, I, THE ARCHIVES INT. MED., 1913, xii, 613.

[16] It may be of interest to note in this connection that efforts made to produce typhoid carriers in Macacus rhesus monkeys by injecting large doses of living culture directly into the circulation have failed.

[17] We wish to express our deepest appreciation to the following physicians who have placed cases at our disposal for this study: Drs. E. N. Ewer, Dudley Smith, George L. Reinle, C. A. DePuy, E. M. Lundegaard, C. A. Queirolo, A. Liliencrantz, Guy H. Liliencrantz, M. L. Emerson, A. F. Clarke, W. H. Irwin, E. A. Majors, H. A. Mackinson, A. M. Shade, C. S. Powell, C. H. Rowe, E. G. Simon, C. R. Krone, F. M. Sylvester, A. S. Kelly, J. M. Shannon, W. H. Streitmann, E. von Adelung and P. F. Abbott of Oakland; Frank W. Simpson, May H. Sampson, Robert Hector, A. M. Meads, L. A. Martin, F. H. Van Tassell, J. J. Benton, J. M. Beuckers, H. S. Delamere, M. F. Toner, R. Paroni, R. T. Legge, H. W. Crane,

We may now proceed to a brief discussion of the results of other investigators who have made use of the recent innovations in the vaccine treatment of typhoid, leaving analysis of details of method and result for comparison with our own records. Recent results with subcutaneous injection of unsensitized vaccine are purposely omitted, however favorable.

Sensitized Typhoid Vaccines Employed Subcutaneously.—Besredka[18] suggested in 1902 the use of dead or living bacteria that had previously been treated with an immune serum, the excess of which was subsequently removed, as means of producing active immunity without severe reaction. In 1911 Metchnikoff and Besredka[19] on the basis of experiments on anthropoid apes, advocated the use of living sensitized typhoid vaccines as the best prophylactic against typhoid fever and their subsequent results[20] would seem to indicate that they are as good as or better than the ordinary vaccine for this purpose. These living sensitized cultures have also been employed subcutaneously in the treatment of typhoid fever during the last few years by several French observers. We find reports by Ardin-Delteil, Nègre and Raynaud,[21] Boinet,[22] Deléarde and Leborgne,[23] Sablé,[24]

Sheffield; W. A. Wood, T. C. McCleave of Berkeley; J. K. Hamilton, R. E. Burns and H. M. Pond of Alameda; H. C. Miller, C. A. Wills of San Leandro; H. C. Moffitt, Wm. P. Lucas, Geo. E. Ebright, P. K. Brown, Chas. A. Pauson, Fred G. Burrows, R. L. Wilbur, G. H. Evans, and R. D. MacKinnon, San Francisco; and L. L. Stanley, San Quentin.

[18] Besredka, A.: De l'immunisation active contre la peste, le choléra et l'infection typhique, Ann. d. l'Inst. Pasteur, 1902, xvi, 918.

[19] Metchnikoff, E., and Besredka, A.: Recherches sur la fièvre typhoïde expérimentale, Ann. de l'Inst. Pasteur, 1911, xxv, 193; Des vaccinations antityphiques, Ibid., p. 865.

[20] Metchnikoff, E., and Besredka, A.: Des vaccinations antityphiques, Ann. de l'Inst. Pasteur, 1913, xxvii, 597.

[21] Ardin-Delteil, Nègre, L., and Raynaud, Maurice: Sur la vaccinotherapie de la fièvre typhoïde, Compt. rend. Acad. d. sc., 1912, clx, 1174; Recherches sur les réactions humorales des malades atteints de fièvre typhoïde traités par le vaccin de Besredka, Compt. rend. Soc. de biol., 1913, lxxiv, 371.

[22] Boinet: Vaccinotherapie de la fièvre typhoïde par le virus sensibilisé de Besredka, Compt. rend. Soc. de biol., 1913, lxxiv, 507.

[23] Deléarde and Leborgne: Province méd., June 21, 1913, p. 273.

[24] Sablé: Jour. d. sc. méd. de Lille, July, 1913, No. 38, p. 25; No. 29, p. 49.

Netter,[25] Roques[26] and Alfaro,[27] Feistmantel[28] and Garbat[29] have employed other preparations of killed sensitized vaccines. In these, as in the later cases, the criteria of improvement differ with individuals and are hard to compare.

Unsensitized Vaccine Administered Intravenously.—Several different preparations of killed typhoid vaccine have been tried intravenously but Vincent's polyvalent ether-killed autolysate has been used in the greatest number of cases. Following ·the brief communication of Thiroloix and Bardon[9] in 1913, articles have appeared by Kraus and Mazza,[10] Kraus,[30] Biedl,[31] Csernel and Marton,[32] Rhein,[33] Reibmayr,[34] Mazza,[35] Holler,[36] Löwy, Lucksch and Wilhelm,[37] Paulicek,[38] Ditt-

[25] Netter: Bull. et mém. Soc. méd. d. hôp., July 24, 1913, p. 126.

[26] Roques: Contribution a l'étude de la vaccinotherapie de la fièvre typhoïde par le virus-vaccin sensibilisé antityphique vivant de Besredka, Thèses University of Toulouse, 1913, Ed. Ch. Dirion.

[27] Alfaro, A.:   Rev. Soc. Med. Argentina, 1913, p. 683.

[28] Feistmantel, C.:   Ueber Prophylaxie und Therapie des Typhus abdominalis mittels Impfstoffen, Wien. klin. Wchnschr., 1915, xxviii, 230.

[29] Garbat, A. L.:   Sensitized Versus Nonsensitized Typhoid Bacteria in the Prophylaxis and Treatment of Typhoid Fever, Jour. Am. Med. Assn., 1915, lxiv, 489.

[30] Kraus, R.:   Bemerkungen ueber Schutzimpfungen und eine Bakteriotherapie des Typhus abdominalis, Wien. klin. Wchnschr., 1914, xxvii, 1443.

[31] Biedl, A.:   (Letter to Paltauf) Zur Vakzinetherapie des Typhus abdominalis, Wien. klin. Wchnschr., 1915, xxviii, 125; Therapeutische Verwendung von Typhus-Impfstoffen beim Menschen, Prag. med. Wchnschr., 1915, xl, 53.

[32] Csernel, E., and Marton, A.:   Die Therapie des Abdominaltyphus mit nicht sensibilisierter Vakzine, Wien. klin. Wchnschr., 1915, xxviii, 229; Die Behandlung des Typhus abdominalis mit nicht sensibilisierter Vakzine, Ibid., p. 733.

[33] Rhein, M.:   Zur Bakteriotherapie des Typhus abdominalis, München. med. Wchnschr., 1915, lxii, 427.

[34] Reibmayr, H.:   Ueber Impfstoffbehandlung des Typhus abdominalis auf intravenösem Wege, München. med. Wchnschr., 1915, lxii, 610.

[35] Mazza, S.:   Die Bakteriotherapie des Typhus abdominalis, Wien. klin. Wchnschr., 1915, xxviii, 64.

[36] Holler, G.:   Zur Vakzinetherapie des Typhus abdominalis, Ztschr. f. klin. Med., 1915, lxxxi, 462; Erfahrungen über Bakteriotherapie des Typhus abdominalis, Med. Klin., 1915, xi, 639 and 668.

[37] Löwy, R., Lucksch, F., and Wilhelm, E.:   Zur Vakzinetherapie des Typhus abdominalis, Wien. klin. Wchnschr., 1915, xxviii, 756.

[38] Paulicek, E.:   Zur Frage der Typhusheilimpfungen, Wien. klin. Wchnschr., 1915, xxviii, 759.

horn and Schultz[39] and McWilliams.[40] · This intravenous injection of typhoid vaccine gives a definite reaction which if certain limits of dosage are exceeded may be alarming or dangerous. We shall consider this reaction in more detail later.

Sensitized Vaccine Administered Intravenously.—So far as we are aware, three types of sensitized typhoid vaccine have been employed for intravenous administration in typhoid fever. Ichikawa,[11] who began this type of treatment (1913), used recent cultures of *B. typhosus* sensitized by the serum of patients recovering from typhoid fever and killed, or at least attenuated, by the addition of phenol. A similar method has been employed by Koranyi.[41] A considerable series of cases have since been treated by Biedl,[31] Eggerth,[42] Sladek and Kotlowski,[43] Boral,[44] Holler,[36] Löwy, Lucksch and Wilhelm,[37] and F. Meyer,[45] who, for the most part, have employed the Besredka living sensitized vaccine.

Any comparison of the results of treatment by these three most recent methods on the basis of percentage benefited, is at best only of suggestive value. As already stated, the criteria on which an estimation of benefit is based vary and no absclute standard is possible, nor do mortality figures offer any conclusive results, but they may be added for completeness since they are at least more definite than any estimated benefit. Since many of the deaths in typhoid are, properly speaking, accidental (hemorrhage, perforations) they can-not be used correctly as criteria of the efficiency of treatment, particu-

[39] Ditthorn, F., and Schultz, W.: Zur Antigenbehandlung des Typhus, Med. Klin., 1915, xi, 100.

[40] McWilliams, H. I.: Treatment of Typhoid Fever with Typhoid Vaccine Administered Intravenously, New York Med. Rec., Oct. 16, 1915, p. 648.

[41] Koranyi, A. V.: Zur Vakzinebehandlung des Typhus abdominalis, Wien. klin. Wchnschr., 1915, xxviii, 85.

[42] Eggerth, H.: Zur Vakzinetherapie des Typhus abdominalis (Letter published by Paltauf), Wien. klin. Wchnschr., 1915, xxviii, 126.

[43] Sladek, J., and Kotlowski, St.: Zur Vakzinetherapie des Typhus abdominalis, Wien. klin. Wchnschr., 1915, xxviii, 389.

[44] Boral, H.: Beitr. zur Frage der Typhustherapie mit Besredka-Vakzine, Wien. klin. Wchnschr., 1915, xxviii, 415.

[45] Meyer, F.: Spezifische Typhusbehandlung, Berl. klin. Wchnschr., 1915, lii, 677.

larly in those patients that are treated fairly late in the disease where such evolving lesions could not reasonably be affected.

TABLE 1.

Summary of Recent Cases of Typhoid Fever Reported as Treated by Newer Methods of Vaccine Treatment.

	Authors.	Number cases.	Benefited.	Mortality.
			per cent	*per cent*
1. Cases treated by subcutaneous injection of sensitized vaccine..................	11	253	57. †	7.1
2. Cases treated by intravenous injection of untreated vaccine....................	14	259	63.	19. *
3. Cases treated by intravenous injection of sensitized vaccine....................	8	207	81.8	9.6

* Due in part to inclusion of a large number of war cases by Paulicek[38] where delayed transportation from the front increased mortality.
† Of 201 cases.

This summary would suggest that the best method of treatment of the three would be the intravenous injection of living sensitized vaccine as based on a higher percentage of benefit and lower mortality than the intravenous injection of unsensitized vaccine. Individual reports would indicate that this difference is really more marked than the summary would show, owing to the fact that the benefit in the third category is more likely to be in the nature of an abortive cure with critical fall of temperature rather than a gradual amelioration and lysis of the fever. Moreover, several authors who have used two or more of the methods do not hesitate unanimously to favor the intravenous over the subcutaneous method and sensitized vaccine over the unsensitized. (Holler,[36] Thiroloix and Bardon,[9] Biedl,[31] Meyer,[45] Löwy, Lucksch and Wilhelm.[37])

Personal Cases.

Owing to the irregular conditions under which our cases occurred in the hospital or home practice of a number of different physicians, it has been necessary for our own records to establish the diagnosis to our own satisfaction by a uniform method. It has also been a pleas-

ure to be able to cooperate in a relatively large number of cases in the differential diagnosis of typhoid fever from other infections, on the basis of laboratory examinations.   Although there is no need of defending the value of laboratory methods as aiding in the diagnosis of typhoid fever, it may still be pertinent to express the value of a complete series of negative examinations as tending to exclude typhoid even in the presence of a temporary clinical appearance of the disease. This is particularly important in view of the fact that the laboratory diagnosis in general practice depends almost entirely on a Widal reaction alone, and that often performed in a not wholly accurate manner.   We wish, incidentally, therefore, to contribute our laboratory results in the differential diagnosis of typhoid, before considering the treatment of those cases in which the diagnosis was positive.

We have carried out laboratory examinations on 105 cases of suspected typhoid fever which may be subdivided as follows:

Total number of cases examined...................................... 105
Cases of proved typhoid... 65
Cases of typhoid not included as treated............................ 12
Cases treated as suggested.. 53

Method of Laboratory Examination.—With few exceptions in the earlier part of the investigation, the routine laboratory examination of each case has been as follows:

1. Blood Culture.—Ten cc. of blood, taken from a vein at the elbow, after proper preparation of the skin with iodin, are mixed with 200 cc. of 10 per cent. bile broth.   In a number of cases the relative intensity of the bacteriemia was estimated by plating 1 or 2 cc. in melted agar (10 cc.).   The broth cultures were examined daily to the tenth day or until positive.   Organisms of correct morphology for the typhoid bacillus were identified by the usual cultural methods in several sugars (glucose and lactose particularly) and in litmus milk.   Final diagnosis of a suspected organism depended on agglutination by an antityphoid serum of high potency.

2. Widal.—Two to 5 cc. of blood were placed in a sterile conical centrifuge tube and allowed to clot.   By centrifugalization the serum could then soon be separated and freed from the blood cells.   A series of dilutions beginning 1:10 in a total volume of 1 cc. of saline was then made running usually to 1:640 or higher when indicated.   To each tube there was then added a drop of a thick standard saline suspension of a stock strain of *B. typhosus* grown on agar bottles in large amount and killed and preserved by the addition of 0.1 per cent. formaldehyde (40 per cent.).   A positive reaction is present in a few hours at room temperature in the lower dilutions, but the final results are read from complete sedi-

mentation of the bacteria with clear supernatant fluid on the following day. This type of macroscopic test gives no pseudo reactions even in a dilution of 1: 10 and very rarely to a prezone of inhibition. This low dilution therefore may be regarded as diagnostic of typhoid fever (except in vaccinated cases).

In the few cases in which both Widal and blood culture were negative, the typhoid bacillus was sought for in stools and urine.

Method of Isolation of B. Typhosus from Stools and Urine.—The well known method of smearing direct from stools on successive litmus lactose agar plates was employed. After incubation, suspicious transparent blue colonies were then transferred to agar and sub-cultures made on glucose and lactose media and on litmus milk. Agglutination tests with antityphoid serum and paratyphoid "a" and "b" serum of known titer were then made. Enriching with 10 per cent. bile broth was also tried, a loopful of the liquid part of the stool or 1 cc. of urine being added to 5 cc. of bile broth. Subsequently plates were made as before.

Cases of Proved Typhoid.—Of the sixty-five cases accepted as typhoid on the basis of laboratory examination, the Widal was positive in sixty (93.7 per cent.), including cases in which the examination was made as early as the fifth day. Of blood cultures taken in fifty-eight of these cases there were forty positive (70 per cent.) including cases taken as late as the thirty-second day.

In One Case Only of This Series Were Both Widal and Blood Culture Negative.—In this case the diagnosis was based on the presence of *B. typhosus* in the stools.

In this series of sixty-five cases are included two cases of infection due to *B. paratyphosus* "b," one of which was treated and one of which refused treatment by the method to be described.

Cases of Proved Typhoid That Were Not Treated.—There are twelve cases among the sixty-five of proved typhoid that were not included among the treated patients for the following reasons:

Cases

No records obtainable from physician	3
Patients died before laboratory diagnosis was complete	2
Post typhoid osteomyelitis	1
Temperature beginning to fall	2
Refused treatment	1
Intravenous injection could not be given	2
Still under treatment	1

These cases are mentioned to emphasize the fact that no choice was exercised in the cases treated. *In every case in which the diagnosis was certainly typhoid the patient was treated if possible except in the*

instances in which a falling temperature might lead to an unwarranted conclusion of benefit produced by the treatment.

There remain forty patients examined in which the clinical diagnosis of suspected typhoid could not be confirmed by laboratory examinations. It is of interest to note that none of these patients gave a positive Widal in dilution of 1:10 by the method described. The blood cultures were negative in all. In five of the clinically more promising cases the stools and urine were also negative. From the point of view of the accuracy of the laboratory examinations it is of interest to note that on the basis of further laboratory examinations and their clinical course, thirty-six could be excluded as definitely not typhoid. From their interest in the matter of differential diagnosis it may be of value to catalogue these cases as follows:

	Cases
Malaria	6
Tuberculosis	4
Brief unknown infections	4
Respiratory infections	4
Trichinosis	2
Influenza	2

and one each of the following: Infection following abortion, erysipelas, tonsillitis, measles, prostatitis, postoperative infection of the nose, appendicitis, pleurisy, coryza, pharyngitis, normal, endocarditis, cystitis, constipation.

There remain then four cases, which, on the basis of excellent clinical evidence alone and in absence of confirmatory laboratory proof, may still from one point of view be classed as typhoid. These cases are not included in our treated series on the ground of not being certainly proved typhoid. It may be remarked, however, that two of these patients were treated and both *recovered abruptly* with a single intravenous injection of our vaccine. They would, therefore, if added to our series, simply increase the percentage of favorable results.

We have then a series of fifty-three cases of typhoid fever in which the clinical diagnosis was fully verified by laboratory examination and in which it was possible to carry out specific treatment as intended. Owing to the extremely variable conditions under which these cases have been studied, both in hospitals and homes, it has been manifestly impossible in many instances to carry out as complete examina-

tions from the laboratory point of view as could be desired. The general conduct of the cases from the point of view of diet, hydrotherapy and other palliative measures, we have not been able, or, indeed, wished to control. The variations, then, from the standpoint of general treatment are precisely those which one might expect from the logically different conceptions of the disease found in any group of fifty physicians. The conduct of the vaccine treatment has, however, owing to the uniform courtesy of those who have allowed us the privilege of this study, been developed from our own experience and the interpretations are again our own. We can only feel that if a similar group of cases could be studied and treated under a uniform set of conditions, that the undoubtedly beneficial results which we attribute to this specific treatment would only be enhanced.

Method of Treatment.

The typhoid vaccine we have used in the treatment of these cases of typhoid fever is one that has already been advocated for the prophylactic immunization of human beings by Gay and Claypole.[14] It consists of the ground sediment of a mixed polyvalent vaccine that has been sensitized by an antityphoid serum and then killed and precipitated by alcohol. From this ground culture the endotoxins are extracted by carbolated saline solution and the remaining sediment of bacterial bodies alone used. On the basis of experimental results this vaccine was found to be superior for protecting rabbits against infection with the typhoid bacillus as compared with other types of vaccine commonly employed, including Besredka's living sensitized vaccine. In the practical use for prophylactic purposes of this vaccine in man it has been shown that it produces less symptomatic disturbance on inoculation (Force[46]) and may be regarded as more protective against typhoid fever in civil communities than several other types of commercial vaccine (Sawyer[47]) as judged by the incidence of typhoid fever among the vaccinated. That the protection afforded by this vaccine employed in civil communities is relative and not so perfect as

[46] Force, J. N.: Institutional Vaccination Against Typhoid Fever, Am. Jour. Pub. Health, 1913, iii, 750.

[47] Sawyer, W. A.: The Efficiency of Various Antityphoid Vaccines, Jour. Am. Med. Assn., 1915, lxv, 1413.

that hitherto obtained in the United States Army we do not regard as proof that this vaccine may not be as superior to other types of vaccine, including Army vaccine, in practical civil conditions of human infection, as our experimental results in animals would lead us to expect.

This polyvalent, sensitized typhoid vaccine sediment is administered for prophylactic purposes in doses of a suspension of 1/10 mg. of dried bacteria, which corresponds to an original bacteria count of about 750 million.   Such a dose when given subcutaneously in a normal individual very rarely produces more than a slight local reaction. For therapeutic purposes we have given doses intravenously ranging from 1/100 to 1/10 mg., most of the doses being between 1/50 and 1/25 mg. (150 to 300 million bacteria).   Such a dose when administered intravenously produces a series of distinct symptoms which vary markedly in intensity with individuals and which are apparently similar in normal and typhoid cases.   Although our symptoms are like those described by recent writers who have used the intravenous method for either sensitized or unsensitized dead cultures, it is reasonable to assume that in aliquot parts our vaccine should be less toxic owing to the abstraction of the endotoxins from the ground bacteria. This lesser toxicity seems to have been proved by our results.   Although the upper range of doses mentioned may provoke alarming symptoms in the more susceptible individuals, it may be stated at once that in over 150 doses which we have administered no eventual or even temporary harm seems to have been done to the patient.   It seems necessary to produce a moderate reaction to bring about the desired results.   Before discussing the symptoms that have been noted we may present the record of a typical *severe* reaction produced in a normal individual who volunteered for the purpose.

S. B. H., male, aged 27, had never had typhoid fever or been vaccinated against typhoid.   In good health.   Oct. 28, 1915, given 180 million sensitized vaccine intravenously.

Leukocyte count before the injection, 6,600; polymorphonuclears, 54 per cent.; lymphocytes, 28 per cent.; large mononuclears, 18 per cent.   Temperature, 100 F.

One half hour after injection patient had a general shaking chill lasting thirty minutes.   Patient was not cyanotic but had slight respiratory distress due to muscle spasm.   Patient vomited twice after chill was over.   Temperature rose gradually to a maximum of 104.4 three hours after injection and then fell to 102.4

at the fifth hour and 97.6 at the tenth hour.   It had returned to 99.2 in twenty hours, where it remained.   Pulse rose to 120.

Leukocyte counts following injection were as follows:

Hours after	No.	Polys.	Lymphs.	Large monos.	Eosin.
		per cent.	*per cent.*		
One..........................	4,200	70	22	8	..
Three........................	7,900	88	6	6	..
Five..........................	8,800	89	6	5	..
Ten..........................	12,000	80	10	10	..
Twelve.......................	12,200	75	11	14	..
Fourteen......................	15,400	71	16	13	..
Sixteen.......................	16,600	52	15	32	1
Eighteen......................	14,000 .	74	8	18	..
Twenty.......................	12,000	60	17	23	..
Twenty-two...................	11,000	50	6	44	..
Thirty-six....................	11,600	25	22	52	1
Sixty........................	11,000	42	27	31	..

This reaction in a normal individual is similar to the one produced in a case of typhoid fever, though distinctly more severe than the average.

A composite picture of the train of symptoms that follows the intravenous injection of our sensitized vaccine given in proper dosage is as follows:

A chill occurs beginning in one half hour to one hour and lasting from a few minutes to ten or fifteen.   This chill is accompanied by a rise in temperature of 1 to 3 degrees, which reaches its height within three hours after injection, and then falls.   There may be a rise of temperature without chill.   The rise in temperature is accompanied by a leukopenia as low as 2,000 to 3,000 per cubic millimeter, which may be preceded by a very transitory hyperleukocytosis during the chill.   The chill is accompanied by an increase in the pulse rate (say to 120).   Slight cyanosis, slight respiratory distress and frequently discomfort may occur.

The fall in temperature reaches normal or subnormal (as low as 94 F. per rectum) in about twelve hours.   This fall in temperature is accompanied by sweating, which may be profuse and last for several hours, relaxation, and usually general amelioration of such symptoms as headache, delirium and the like.   The patient often feels perfectly

well and demands food and even when this condition is transitory it would seem to be beneficial. Coincidently there is a rise in leukocytes which may reach as high as 40,000 and frequently 15,000 to 20,000. This is represented by a relative polymorphonuclear increase of 80 to 90 per cent. This hyperleukocytosis is the more striking in view of the characteristic leukopenia of typhoid. This particular reaction, which we regard as of peculiar significance, was predictable from our experimental results in rabbits and mentioned in a preliminary communication.[48] It has since been touched on by Holler[36] and confirmed fully by McWilliams.[40]

In four instances we feel that the dose administered was too large for the individual case and in such instances there may be vomiting, cyanosis and even symptoms of collapse, with irregular heart action. In one case in which 800 million were given by another's mistake, there was partial collapse and small hemorrhages in the mouth reported.

The possible dangers of the intravenous method of treatment, whether with unsensitized or with sensitized vaccines, have been mentioned by a number of recent authors. It should be stated that unfavorable reports are based on the use of vaccines more toxic than the one we have employed and the criticisms when severe have been based on a very limited experience. Thus Boral[44] had a single patient who died three days after intravenous injection of the Besredka vaccine, in which case the outcome was attributed, for no specific reason, to the vaccine. Sladek and Kotlowski[43] urge caution owing to possible danger of collapse. Deutsch[49] had one patient who died with meningeal symptoms five days after treatment. Biedl[31] noted increase of epistaxis in two of his patients. Csernel and Marton[32] believe the intravenous injections are contraindicated in hemorrhage, perforation, cholecystitis and cases with irregular heart action. It seems evident that with growing experience the danger decreases (Koranyi[41]) and we are inclined to believe with Holler[36] that the danger lies not so much in the method as in the physician who administers the vaccine. Of the authors mentioned, it is interesting to note that only one (Deutsch) really abandoned the intravenous route.

[48] Gay, F. P.: Abortive Treatment of Typhoid Fever by Sensitized Typhoid Vaccine Sediment, Jour. Am. Med. Assn., 1915, lxv, 322.

[49] Deutsch, F.: Zur Vakzinebehandlung des Typhus abdominalis, Wien. klin. Wchnschr., 1915, xxviii, 810.

It would seem evident from the symptoms that we have described that great caution should be used in choosing the dose to be employed with particular regard to the existence of such existing complications as abnormal cardiac functioning and hemorrhage. We have not seen any contraindication in the presence of slight bronchial or bronchopneumonic involvement and have proceeded cautiously with the treatment in four such cases. We have further treated one patient in whom hemorrhage had begun without increasing it.

No detailed method of procedure can be prescribed in treating a given case of typhoid fever by our sensitized sediment. The best results seem to be obtained by provoking a distinct but not too severe reaction of the type outlined, characterized particularly by a temperature excursion and hyperleukocytosis. The dose necessary to produce such a result varies markedly with the individual and with the particular balance already established between the infecting agent and resisting host. A single injection may be all that is necessary to restore the individual to an essentially normal condition as judged from the temperature chart; and as has been mentioned, the subjective symptoms follow the fever. The temperature may drop to normal following the initial rise and remain there, in which case no further injections are necessary, except perhaps to prevent relapse. As a rule, however, the most favorable type of rapid return to normal is a matter of two or three days instead of twenty-four hours, and we have usually waited this longer period before repeating the treatment.

We had best leave further discussion of the variations in method of treatment until we have given our results as a whole, together with such correlations as present themselves between the results produced and the blood findings before and after injection.

Our cases may be readily divided in respect to results, into three rather sharply defined groups, which we herewith exemplify by the appended type charts. (Figs. 1–3.)

Group 1. Relatively Unaffected Cases.—This group comprises eighteen, or 34 per cent. of our cases. Each and all of the successive treatments in these cases, although frequently resulting in temperature excursion, hyperleukocytosis, and the other symptoms, apparently produce no permanent result so far as shortening the course of the disease is concerned. It is not strictly speaking exact to classify

these as "unaffected" for, as mentioned, even the temporary ameliorations in temperature and subjective symptoms we are inclined to regard as beneficial. In a number of these cases it will be seen from the remarks on Table 2 that the fever ran permanently lower after treatment. In other cases the bacteria were diminished or disappeared from the blood. It is, moreover, distinctly to be noted that in none of these cases did the use of vaccine apparently weaken the patient or contribute in any demonstrable way to a fatal outcome when such occurred. (Fig. 1, Case 47.)
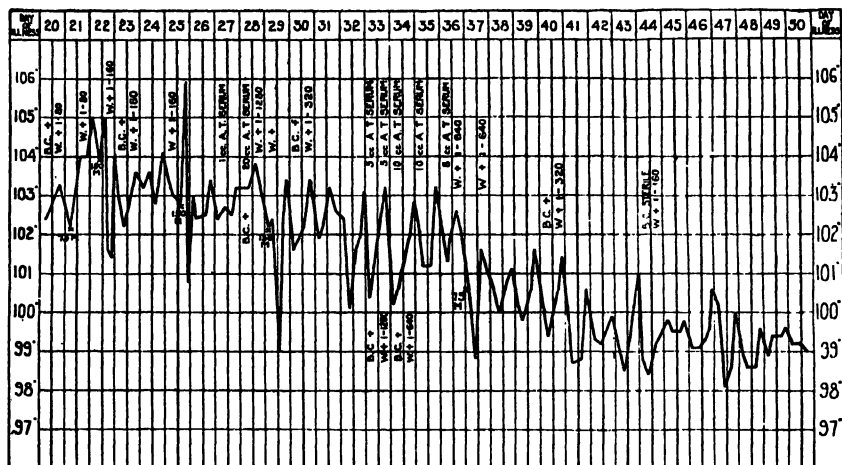
Fig. 1.—Temperature curve and other data in Case 47. The data in these charts are abbreviated as follows: B. C. = Blood culture; W. = Widal; A. T. Serum = Antityphoid serum; 75 M = Sensitized vaccine corresponding to 75 million typhoid bacilli.

Group 2. Benefited Cases.—Of these cases there were thirteen, or 24.5 per cent., in our series. In these cases one or more doses of vaccine led not only to temporary amelioration but to a lytic type of defervescence, the successive drops in temperature being related directly to the vaccine treatments. In cases of this group the duration of the disease was apparently shortened and the course markedly ameliorated. (Fig. 2, Case 43.)

Group 3. Aborted Cases.—Cases totaling twenty-two, or 41.5 per cent., of our series. In these cases the injection of vaccine led to a more or less critical fall of temperature directly associated with the vaccine injection. (Fig. 3, Case 35.)

TABLE 2.

Relatively Unaffected Cases.

No.	Age.	Widal.	Blood culture.	Treatment, day.	Number of treatments.	Permanent normal temp.,* day.	Remarks.
2	14	0	0	14	7	Died 32d day	Severe case complicated by laryngeal symptoms. Necropsy refused.
6	20	+80	+	6	4	38	Severe case. White count rose from 8,000 to 21,000 after vaccine treatment.
11	7	+	+	5	5	28	Severe case. Delirium. Abdominal pain.
12	5	+	+	7	4	29	Severe case. Delirium. Abdominal pain.
13	25	+	+	15	3	30	Severe case. Temperature distinctly lower following vaccine treatment, but course of disease not shortened.
14	20	+50	+	15	5	48	Severe case. No effect on temperature course.
16	45	+	+	12	5	49	Severe case. No effect on temperature course.
18	30	+320	+	15	4	45	Severe case. Temporary effect on temperature course.
20	35	0	+	7	2	Died 10th day	Severe. Very toxic. Patient unconscious when first seen.
44	22	+40	+	14	4	52	Moderately severe. Slight symptomatic improvement following injection of vaccine.
47	62	+80	+	21	6	48	Severe case. Blood culture positive to fortieth day of disease.
48	14	0	+	21	1	Died 26th day	Severe case. Toxic. Organism from blood at first inagglutinable. Exsanguinating hemorrhage.
57	56	+1,280†	+	21	1	Died 27th day	Severe case. Temperature normal after vaccine treatment. Died six days later from hemorrhage and perforation.
61	19	+320	+	14	5	43	Severe case. Muscle spasm. One colony B. typhosus per cc. blood on twenty-second day. Blood sterile three days later following combined vaccine and serum treatment.

* A temperature below 100 F. by rectum for entire day.

† This case with high Widal is not an exception. The first injection of vaccine brought the patient's temperature down about three degrees during three days. He died three days later of perforation. This Widal titer is therefore not included in the average.

TABLE 2—*Concluded.*

No.	Age.	Widal.	Blood culture.	Treat-ment, day.	Number of treat-ments.	Permanent normal temp.,* day.	Remarks.
66	55	+40	+	14	4	Died 30th day	Severe case. Muscle spasm. Blood culture positive on twenty-fifth day of disease. Three hours after hemorrhage and perforation took place, patient was operated on and perforation closed. Patient's general condition was so serious that death followed in spite of there being practically no gross soiling of the peritoneal cavity.
72	20	+160	+	11	4	57	Severe case; three colonies B. typhosus in 1 cc. blood on eleventh day. Blood sterile ten days later, following serum and vaccine treatment. Relapse began on thirty-first day, lasting until fifty-seventh day.
75	46	+80	+	15	5	35	Severe case; one colony B. typhosus in 2 cc. blood on nineteenth day. Positive with 1 cc. blood until twenty-eighth day. Blood sterile on thirty-first day and defervescence occurred four days later. Hyperleukocytic crisis 17,000 to 18,000 after vaccine treatment.
79	19	+320	+	5	4	29	Severe case. Toxic and comatose. Symptomatically improved by treatment but course not notably shortened.
	28	+114	95%	13	4	41	

* A temperature below 100 F. by rectum for entire day

TABLE 3.

Benefited Cases.

No.	Age.	Widal.	Blood culture.	Treat-ment, day.	Number of treat-ments.	Perma-nent normal temp., day.	Dura-tion of treat-ment, days.	Remarks.
23	21	+160	+	6	2	21	15	Laboratory infection. Blood culture positive fourth day. Symptomatically well on thirteenth day. Afternoon rise in temperature for several days thereafter.
32	10	+1,280	+	11	3	32	21	Severe case. Very toxic. Treatment very cautious because of slight intestinal hemorrhage.
38	18	+40	+	9	3	27	18	Severe case. Toxic, with muscular spasticity. Blood culture positive after three vaccine treatments.
39	8	+160	+	11	5	30	19	Severe case. Complicated by pyelitis. W. B. C. rose to from 10,300 to 25,000 after each vaccine treatment.
42	23	+160	+	13	4	26	13	Moderately severe. Marked diarrhea. W. B. C. rose from 7,800 to 16,250 after vaccine treatment.
43	25	+80	0	16	2	26	10	Mild case.
46	24	+40	+	11	3	28	17	Severe case. Blood culture sterile three days after combined vaccine and serum treatment.
49	28	+40	+	15	3	34	19	Severe case. Blood culture sterile 72 hours after first vaccine treatment. Symptomatically well twenty-third day. Leukocytes rose from 7,400 to 15,200 after treatment.
50	7	+40	+	16	1	28	12	Severe case. Toxic and distended.
74	26	+160	+	18	3	30	12	Moderately severe. Blood culture positive twenty-second day. Hyperleukocytic crisis of 15,200 after vaccine treatment.
81	35	+80	0	11	3	21	10	Mild case.
94	30	+80	+	7	3	23	16	Moderately severe. Blood culture sterile four days after second vaccine treatment.
104	23	+50	+	18	2	28	10	Moderately severe.
	21	+182	84.6%	12.4	2.8	27	14.7	

TABLE 4.

Abortively Recovered Cases.

No.	Age.	Widal.	Blood culture.	Treatment, day.	Number of treatments.	Permanent normal temp., day.	Duration of treatment, days.	Remarks.
1	70	+	....	12	1	18	6	Temperature had ranged from 100 to 103 for ten days previous to vaccine treatment.
5	30	+1–80	0	5	3	21	16	Severe case. Hyperleukocytosis of 20,200 following injection of vaccine.
10	14	+	....	10	1	16	6	Moderately severe case.
21	53	+640	0	15	2	21	6	Mild.
26	14	+	....	16	2	23	7	Mild.
27	38	+160	+	10	3	19	9	Blood cultures sterile 24 and 96 hours after vaccine treatment. W. B. C. 4,000 to 14,000.
31	19	+320 ·	0	10	1	15	5	Mild.
33	15	+320	0	15	2	24	9	Moderately severe.
34	6	+320	0	14	1	18	4	Mild.
35	28	+160	+	7	2	13	6	Blood cultures sterile 24 and 48 hours after vaccine treatment.
40	20	+640	0	14	2	22	8	Mild. Accompanied by profuse perspiration.
41	8	+160	0	18	2	26	8	Mild.
51	5	∓20	0	6	1	8	2	Mild. Two of three children ill at same time. Blood culture positive in one case.
52	4	+10	0	6	1	11	5	Mild. Two of three children ill at same time. Blood culture positive in one case.
59	5	+20	+	10	2	16	6	Moderately severe. Blood culture sterile four days after first treatment.
63	38	+160	0	17	2	23	6	Moderately severe.
70	21	+160	+	8	3	18	10	Delirious, severe headache. Blood culture sterile 48 hours after first treatment.
73	30	+1,280	+	32	3	40	8	Comatose and involuntary. Pulse 130. Condition critical.
80	14	+160	0	26	1	35	9	Moderately severe.
90	8	+40	+	10	2	23	13	Blood culture sterile five days after first treatment. None taken between.
95	25	+	+	12	2	21	9	Moderately severe.
103*	30	+1–40	+	8	3	11	3	Severe.
	22.5	260	42%	12.7	2–	20	7.3	

* Case of paratyphoid "B."

A consideration of our cases as arranged in these three groups brings out a number of interesting correlations. (Tables 2, 3 and 4.)

We see, for instance, that the treatment was begun on the average on about the same day in all three categories (12 to 13) and that the average age of the patients was nearly the same. The first group of "Relatively Unaffected Cases" differs distinctly from the "Abortively Recovered" ones in that the blood cultures were positive in over twice
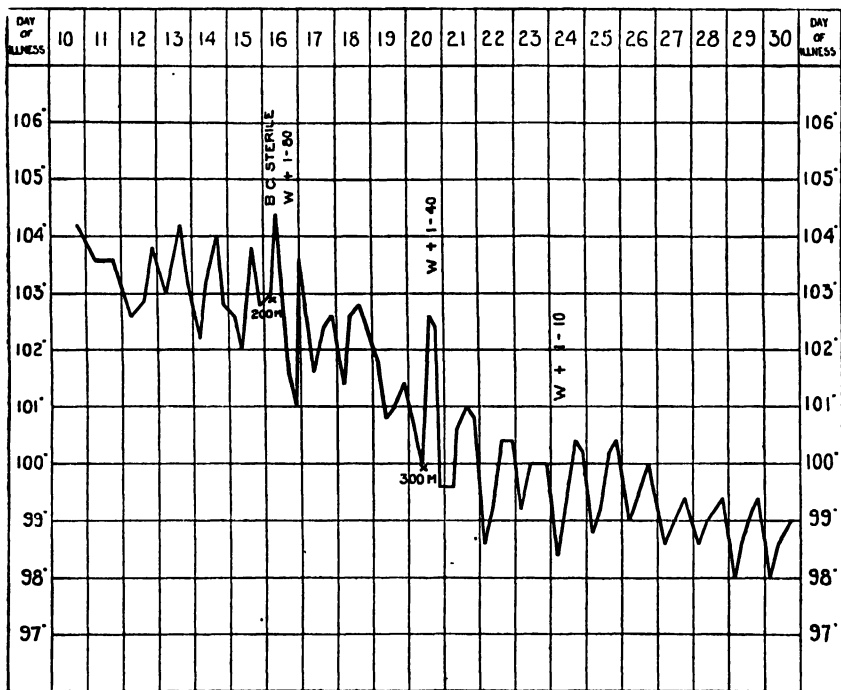
Fig. 2.—Temperature curve and other data in Case 43.

as many cases in the first (95 per cent. as compared with 42 per cent.) and again in the fact that the Widal was much lower on the average in the unaffected cases than in the aborted cases (1 to 114 and 1 to 260). The benefited cases (Group 2) lie intermediate between the two, having nearly as high a percentage of blood cultures as Group 1 and nearly as high a Widal average as Group 3.

There is little doubt that the number of typhoid bacilli in the circulating blood bears a distinct relation to the severity of the course of

the disease, and the persistence of positive blood cultures indicates an unfavorable outcome (see particularly Schottmüller,[50] Jochmann[51]). Conversely the disappearance of the bacteriemia early in the disease is a favorable indication.   From such facts we should expect to find the third group of cases in a general way milder than Group 1.
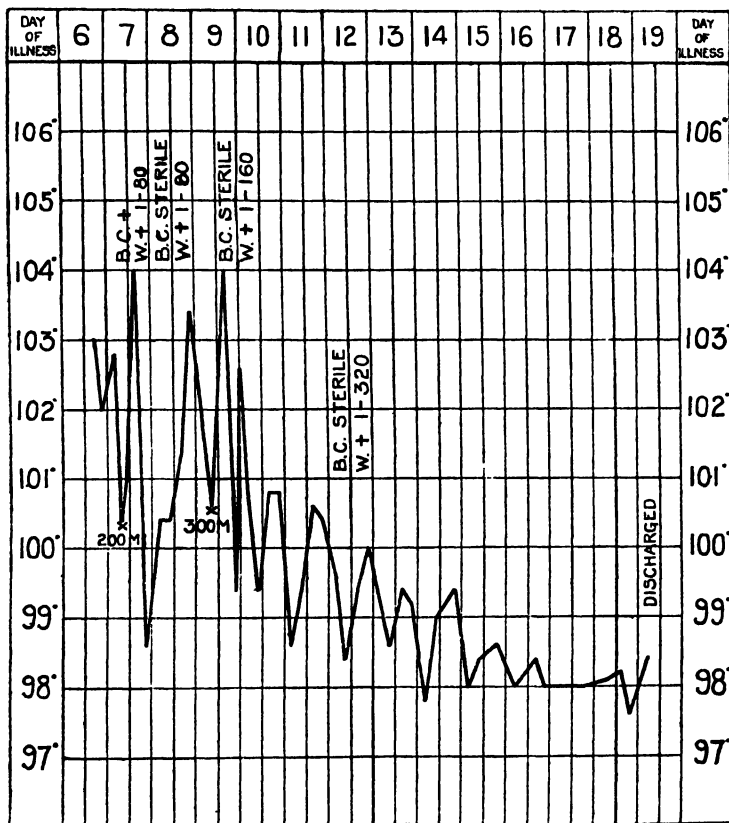
Fig. 3.—Temperature curve and other data in Case 35.

Whereas it is impossible to judge what the outcome of any one of our cases would have been without treatment, certain impressions of the severity of the cases *before treatment* in relation to the outcome may be of interest (Table 5).

[50] Schottmüller, H.:   Die typhösen Erkrankungen, Handb. der inn. Med., 1912, I, Mohr and Staehelin.
[51] Jochmann: Lehrbuch der Infektionskrankheiten, Springer, Berlin, 1914.

TABLE 5.

General Condition of the Cases before Treatment.

	Severe.	Moderate.	Mild.
Group 1, unaffected......................	15	3	0
Group 2, benefited......................	6	5	2
Group 3, aborted.......................	4	7	11
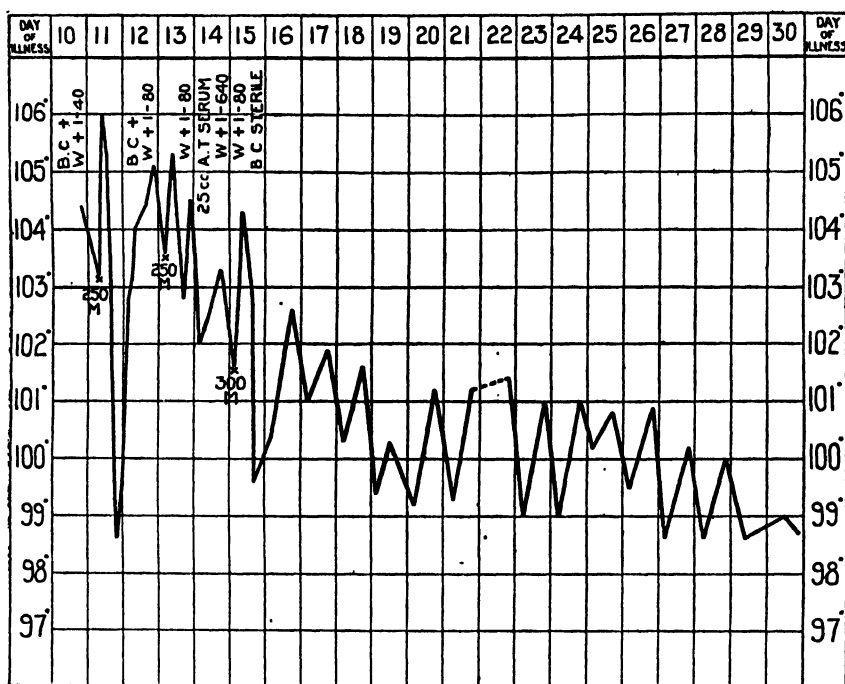
Fig. 4.—Temperature curve and other data in Case 46.

It is evident that the milder cases were more likely to be favorably affected by treatment than the severe ones. In this connection it may be wise to forestall criticisms that might be made to our interpretation of the abrupt recoveries as due to the injection of vaccine. It may be suggested by clinicians with an extensive experience in typhoid fever that they have seen cases (usually a case) in which the temperature fell by crisis in typhoid fever without treatment. We have been able to find little detailed account of such a course in the

literature. McCrae[52] in his carefully analyzed series of 1,500 cases noted a critical fall in two cases only (0.1 per cent.). He further notes a mild form of typhoid in 44 cases (3 per cent.). These figures added or separately, may be compared with our 41.5 per cent. of rapid recoveries. Of further significance is the direct relation of the vaccine injection to the result produced and the fact that the average duration of the specific treatment until a *permanent* normal temperature was reached was a little over seven days.

In this connection it may be of interest to contrast the average duration of the disease in the three categories as compared with McCrae's figures (Table 6).

TABLE 6.

Duration of Disease as Contrasted with McCrae's Figures.

Group 1, unaffected.. 41   days
Group 2, benefited... 27   days
Group 3, aborted... 20   days
_____
Real average.. 27.6 days
Average of McCrae's 1,500 cases........................... 31   days

The relative intensity of the Widal reaction in the favorably affected cases as compared with the others seems to us of peculiar significance. It may be taken as an indication of the degree of successful response that the patient has made to the infection. We do not wish to assume that the agglutinin titer is any true measure of resistance, but it often runs parallel to those antibodies that are responsible for tipping the balance in the patient's favor. Our conception of the recovery that is favored by the intravenous injection of sensitized vaccine would be that it is due first to a hyperleukocytosis produced in maximum degree by the use of tropinized bacteria, and secondly to the action of the patient's own antibodies on the circulating bacteria. Such a conception to be proved would necessitate an estimation of the patient's tropins in relation to results produced rather than the agglutinins. The establishment of an accurate method for such determinations is at present engaging our attention.

A further corollary of this hypothesis should show that the bacteria in the patient's blood disappear or diminish in the presence of a

[52] McCrae: Osler's System of Medicine, Ed. 1, ii, 70.

hyperleukocytic rise and the simultaneous presence of suitable anti-bodies. This was found to be the case in rabbits in the experiments of Gay and Claypole. Although it has not been possible to take an extensive series of blood cultures in many of our cases, our results show that in ten cases positive blood cultures became sterile in from one to three days after injection of vaccine. We contemplate a more extensive series in this direction. In addition to diminishing the bac-teriemia or actually sterilizing the patients, as would seem to be the case in many of our abortively recovered cases, the injection of sen-sitized vaccine in the majority of cases is accompanied by an increase of the Widal titer which, as we have seen, is of favorable prognostic significance.

In view of the probable mechanism of recovery in typhoid fever induced by the intravenous injection of sensitized vaccine, it might seem reasonable in those cases associated with a low Widal titer in which less favorable results may be anticipated, to supply the neces-sary antibodies artificially. This could be done by the use of an im-mune serum. One is not impressed with the results hitherto attained in the serum therapy of typhoid (Chantemesse,[53] Rodet,[54] Rodet and Lagriffoul,[55] Lüdke,[56] Andriescu and Ciuca,[57] and Koenigsfeld[58]). We have no certainty whether such a serum should be antiendotoxic, or according to our own working hypothesis, largely tropic. While wait-ing for further experimental evidence on this point we have felt jus-tified in certain of our cases, particularly in those with a low Widal, in

[53] Chantemesse, M.: Toxine typhoïde soluble et sérum antitoxique de la fièvre typhoïde, Prog. méd., 1898, Series 3, vii, 245.

[54] Rodet, A.: Die Serotherapie beim Typhus, Handb. der Serumtherapie, A. Wolf-Eisner, München, 1910.

[55] Rodet, A., and Lagriffoul: La sérothérapie de la fièvre typhoïde, Presse méd., 1910, xviii, 969.

[56] Lüdke, H.: Die Serumtherapie des Abdominaltyphus, München. med. Wchnschr., 1912, xxv, 907; Die Behandlung des Abdominaltyphus mit intravenö-sen Injektionen von Albumosen, München. med. Wchnschr., 1915, xxviii, 321.

[57] Andriescu, C., and Ciuca, M.: De l'action du sérum antityphique de Bes-redka sur l'évolution de la fièvre typhoïde, Ann. de l'Inst. Pasteur, 1913, xxvii, 170.

[58] Koenigsfeld, H.: Ein neues Prinzip der Serumtherapie bei Infektionskrank-heiten mit besonderer Berucksichtigung des Typhus abdominalis, München. med. Wchnschr., 1915, lxii, 253.

giving intravenous injections of the serum of goats that had received repeated subcutaneous and intravenous injections of several strains of living *B. typhosus*. Whereas the result with such serums has not been in many instances striking, it has at all events shown the harmlessness of goat serum even when given in fairly large quantities intravenously.

Fresh goat serum was first shown to have no hemolytic or hemagglutinative effect on human blood corpuscles. In each case where intravenous injection was intended 1 cc. of goat serum (either normal or immune) was given subcutaneously twenty-four hours previously to avoid possible anaphylactic shock. In all eight cases were given intravenous injections ranging from 20 to 95 cc. In no instance was any untoward immediate symptom noted, and in only one case was a slight urticaria found subsequently. This latter finding may be contrasted with the usual results which follow the intravenous injection of large amounts of immune serums from the horse (Cole[59]). In all instances, as expected, the infusion of a relatively considerable amount of antiserum caused an immediate rise of the agglutinins in the patient and in several instances the serum injection alone seemed followed by a symptomatic improvement and slight defervescence.

In one case (Fig. 4, Case 46) which we trust will not prove to be exceptional, two injections of vaccine in a patient with low Widal and positive culture early in the disease (tenth day) were followed by persistent positive cultures and only temporary temperature fall. The administration of 25 cc. of antityphoid serum intravenously followed the next day by a third dose of vaccine, led to sterilization of the blood stream and immediate defervescence. In this case at least it seems evident that the cure was due to the mixed serum and vaccine treatment.

Fatal Cases.

It will be seen from Table 2, comprising our relatively unaffected cases, that there have been five fatal cases in our treated series, or a little over 9 per cent., which does not differ from McCrae's large series with the same mortality. Of course no mortality average is of

[59] Cole, R.: Pneumococcus Infection and Lobar Pneumonia, THE ARCHIVES INT. MED., 1914, xiv, 56.

great significance in so small a series as our own.   It is to be noted that a disproportionate number of our cases have died from the "accidents" of typhoid, if we may so call them.   There was hemorrhage in three cases, two of which were followed by perforation.   These two complications should account for death in 40 per cent. of the cases according to McCrae.   In our own series they represent 60 per cent. of the fatalities.   It should be noted that in two of these cases the treatment was not begun until the twenty-first day.   True typhoid toxemia was the cause of death in only one of our cases (20).   Case 2 was toxic, but probably died of some laryngeal complication; necropsy was refused.

Relapses.

There have been five relapses in our series (9 per cent.), somewhat lower than McCrae's average of 11.4 per cent.   It should be noted that two relapses have occurred in our cases listed as abortively recovered after discharge from the hospital; both promptly responded to a single reinjection of vaccine.   The early discharge of the patient we believe had a distinct influence on the occurrence of these relapses.   During the latter part of our series we have followed the intravenous treatments with a series of three subcutaneous injections of the vaccine in the dosage ordinarily employed for prophylaxis (1/10 mg. = 750 million).   In twelve cases in which this treatment has been fully carried out there have been no relapses, whereas the five relapses occurred in forty-one cases in which no such treatment was employed or completed.   These subsequent subcutaneous injections may be followed by slight rise of temperature.

Specificity of Treatment Employed.

In view of the relative success that has been reported by means of certain nonspecific methods of treating typhoid fever, it may be well to express our conception of the relation of our method of treatment to such methods.

Early in the history of the treatment of typhoid by vaccines Rumpf[2] questioned the specificity of the results claimed by Fraenkel[1] on the ground that he had obtained similar favorable effects by the use of preparations of B. pyocyaneus.   That such results were at least rela-

tively less effective would seem to be shown from the subsequent work of Kraus and Buswell[3] and Presser.[4] Kraus[60] has obtained abortive cures in typhoid by the use of colon vaccines. Ichikawa has cured paratyphoid, as we did, by sensitized typhoid vaccine. Lüdke[55] has mentioned favorable results by the simple use of a deutero-albumose. Letulle and Mage[61] and Gay[62] have treated cases of typhoid by means of a preparation of colloidal gold (colibiase). We believe such favorable results, which we are quite ready to accept, are in reality in perfect harmony with each other and with our own choice of treatment. All of these substances including colloidal gold[63] produce hyperleukocytosis, particularly when injected into the circulation. Leukocytic extracts probably act in the same way in those cases in which they are undoubtedly effective. Any of these substances could therefore be expected to cure a percentage of cases of typhoid owing to the dual mechanism of a hyperleukocytosis plus antibodies in the patient.

Intravenous treatment by means of sensitized typhoid bacilli differs from the above methods in two ways:

1. Owing to the fact that the injected protein is sensitized or tropinized, the response on the part of the leukocytes is much more intense and effective (Gay and Claypole[12]).

2. Typhoid vaccine aids in building up the active immunity of the patient against typhoid fever as no other preparation can.

SUMMARY.

This article deals with the study of 105 cases of suspected typhoid fever in which we were allowed to examine the patients through the great courtesy of a number of physicians in Alameda and San Francisco Counties. Thorough laboratory examinations in most of these cases by blood cultures and Widal tests and in a number the search for the typhoid bacilli in the stools and urine, offer certain facts of

[60] Kraus, R.: Ueber Bakteriotherapie akuter Infektionskrankheiten, Wien. klin. Wchnschr., 1915, xxviii, 29.

[61] Letulle, M., and Mage: Traitement la fièvre typhoïde par l'or colloidal en injections intraveineuses, Bull. de l'Acad. méd., Paris, 1914, lxxii, 421.

[62] Gay: Un traitement pratique de la fièvre typhoïde aux armées, Presse méd., March 4, 1915, p. 67.

[63] See discussion by Robin and Chantemesse in loc. cit., Ref. 60.

interest in the differential diagnosis of typhoid fever. In 65 of the 105 cases the diagnosis of typhoid fever was made from both clinical and laboratory data. In these 65 cases the Widal was positive in 60 (93.7 per cent.) and as early as the fifth day, the high percentage of results being due, in a large measure, to the method employed, namely, the use of the macroscopic method and a formaldehydized culture of the typhoid bacillus. Of the blood cultures taken in 58 cases there were 40 positive (70 per cent.), including a case first seen on the thirty-second day. In only one case of the 65 were both Widal and blood culture negative, which case was diagnosed by the presence of *B. typhosus* in the stools.

Of the 40 cases excluded as not being typhoid fever on a laboratory basis, 36 could ultimately be excluded on both clinical and further laboratory examination. There remain 4 cases which on clinical evidence alone may have been typhoid, but which are not included in our series owing to the fact that laboratory proof was lacking. It may incidentally be remarked that 2 of these cases were treated by the method described, with abrupt recovery. Of the 65 cases it was impossible for various reasons that are stated, to treat 12. The remainder of the cases, however, were all treated without choice.

There remain, then, a series of fifty-three cases of typhoid fever in which the diagnosis was absolutely certain both on clinical and laboratory grounds. We have not attempted to influence the ordinary symptomatic treatment of these cases, which differs as much as might be expected in a group of over fifty physicians. The patients, moreover, were cared for under varying conditions in private homes and hospitals, some of them even without the attention of a trained nurse. This variation in care and location of the cases has undoubtedly an effect on the mortality and has made thorough laboratory examinations, such as leukocyte counts at frequent intervals, impossible in all the cases.

The mortality in these cases has been precisely what one would expect under the best hospital conditions (McCrae), namely 9 per cent., which we regard as suggesting that, under uniform conditions, with our method of treatment the mortality would have been less than the average. The mortality has been composed, to a large extent, of what may be termed the "accidents" of typhoid, namely 60 per cent. of the deaths by hemorrhage or perforation.

Our method of treatment has consisted in the intravenous injection of 1/50 to 1/25 milligram (150 to 300 million bacteria) of a sensitized, polyvalent, killed typhoid vaccine sediment prepared after the method of Gay and Claypole. This injection gives rise to a series of symptoms characterized particularly by a chill, rise and fall of temperature and leukopenia followed by hyperleukocytosis. The fall of temperature with its attendant hyperleukocytosis leaves the patient at least temporarily benefited, and the benefit and normal temperature may be permanent. Thus in 66 per cent. of the cases a distinct benefit was obtained, as shown by lowered temperature, disappearance or amelioration of subjective symptoms and an apparently accelerated recovery. In 41.5 per cent. of this 66 per cent. the recovery was of an abortive form with a critical fall of temperature and a permanent normal temperature established within a few days. This permanent normal temperature was reached on an average seven days after beginning treatment in these cases. There remains, however, 34 per cent. of cases which are classified as relatively unaffected. We regard this classification as underestimating the beneficial results for reasons given. In none of the cases did the use of the vaccine have any apparent harmful effect on the case, although in four, in which too large a dose was used, the symptoms were somewhat alarming.

A series of subcutaneous injections following the intravenous treatment apparently aids in preventing relapses.

We regard the mechanism of benefit and cure in these cases which were affected by the treatment as due to a combination of specific hyperleukocytosis and the presence of antibodies (tropins?) in the patient's blood. The injection of vaccine could be shown in a number of cases to be followed by the disappearance or diminution of bacteriemia and usually also by an increase in the Widal. In those cases which did best the Widal was originally high and those cases which showed the least effect had the low Widals. The cases judged as "mild" before treatment began did better on the whole than those regarded as "severe." There were, however, a number of severe cases which showed abrupt recovery or benefit.

On the hypothesis that successful results are due to the strength of the antibodies already established in the patient, we have ventured in severe cases with low antibody content, to combine with the vaccine

treatment the intravenous injection of considerable amounts of typhoid immune serum from goats.   These cases, although few in number, suggest that this type of treatment with further elaboration might increase the percentage of favorable results.

We regard the use of sensitized vaccine as being better for intravenous injection than plain typhoid vaccine or less specific methods of treatment that have been suggested by other authors, owing to the fact that sensitized typhoid vaccine produces a specific form of hyperleukocytosis of maximum degree (Gay and Claypole), and may also be shown to be followed by an increase in active immunity of the patient against the disease.

THE FASTING TREATMENT OF DIABETES MELLITUS, WITH SPECIAL REFERENCE TO ACIDOSIS.[1]

BY EDGAR STILLMAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Since its inception by Rollo the dietetic treatment of diabetes mellitus has had as its object the attainment of the aglycosuric state. To attain this state, carbohydrate was withdrawn from the diet; this was done, but never quite satisfactorily, because withdrawal of carbohydrate from the diet, it was believed, precipitated acidosis. And because it was supposed to precipitate acidosis, prolonged fasting was considered inadvisable, although it was recognized that the occasional fast days of Naunyn diminish acidosis. This procedure was not followed, because it was thought that "fats burn in the fuel of carbohydrate." The spirit of this phrase has dominated the modern treatment of acidosis. The doctrine that overfeeding with fat is harmless and even beneficial in increasing weight and strength and that carbohydrate feeding is required to avert threatening coma has done much to retard the progress of the treatment of diabetes. In point of fact the excess of fat in diet disposes the patient to acidosis; the excess of carbohydrate to glycosuria. Now it has been found that both features of the disease—glycosuria and acidosis—must be recognized and controlled. Both can be influenced by diet; both can be influenced by fasting. In order that treatment by these means may be properly applied it is necessary to understand the effect of diet and fasting upon both glycosuria and acidosis. As far as glycosuria is concerned a few days of complete fast may render the patient sugar-free and a subsequent restriction of diet may suffice to maintain him in this state; but the control of acidosis is more difficult and involves a comprehensive understanding of the problem of acidosis in general. It is, indeed, the presence or absence of this condition which is now regarded as the dominant factor in determining the

[1] Read before the New York Academy of Medicine, December 21, 1915.

course of treatment.   In view of this fact the present paper is limited to a discussion of this phase of the subject.

Acidosis.

By acidosis is meant a decreased alkaline reserve of the blood rather than an increased acidity.   An accumulation of acids sufficient to create an acid reaction in the blood and tissues is incompatible with life.   The alkalinity of the blood is maintained chiefly by the amount of bicarbonate it contains.   This amount may be estimated indirectly in terms of carbon-dioxide tension in the alveolar air, directly by the power of the blood plasma to combine with carbon dioxide.   If acids accumulate in the blood and tissues sufficient to neutralize a portion of the bicarbonate there present, they reduce the alkaline reserve of the blood and tissues below normal.   When this occurs it is accompanied by a diminution in the power of the blood plasma to combine with carbon dioxide and consequently causes a parallel diminution in the tension of alveolar carbon dioxide.   This condition is known as acidosis, although an acid reaction of the blood never occurs unless *in extremis.*   Acidosis, therefore, may be defined as a lessened bicarbonate reserve in the blood and tissues.   It may be caused either by an overproduction of acid bodies in metabolism or by a lessened excretion of these substances.

The need of a method to determine the amount of acid bodies in the medium where accumulation takes place is obvious.   That medium is the blood.   Tests of the urine are valuable only for the excretory phase of acidosis.   They may be misleading in that they give no index of the accumulation of these substances in the blood due to overproduction or to faulty elimination.

Methods for Determining Acidosis.—Until recently urinary tests alone have been used as indices of acidosis.   These tests have included qualitative tests for acetone and diacetic acid; quantitative determinations of the acetone bodies, of ammonia, and of total acidity.   So long as excretion keeps pace with acid formation, tests of this nature are satisfactory; but when excretion of acid substances is imperfect, as may occur in diabetes, the elimination cannot keep pace with production.   Therefore urinary tests may not reveal the degree of acido-

sis present. During the past thirteen years Beddard, Pembrey, and Spriggs[2] have published a series of papers on the amount of carbon dioxide in the blood, the urine, and the alveolar air as criteria of acidosis in diabetes. Since 1913[3] they have abandoned blood analyses in favor of determinations of the alveolar carbon dioxide, because their studies led them to believe that "estimations of alveolar carbon dioxide by Haldane's method are at present the best guide for the prognosis and treatment of diabetes." The alveolar carbon dioxide, being an indirect measure of the bicarbonate content of the blood, is significant, but in abandoning examination of the blood the English investigators have substituted an indirect measure of acidosis for a direct one. The indirect method, namely, the alveolar carbon-dioxide determination, is an accurate indicator of the reserve bicarbonate of the blood only when respiration and circulation behave in an entirely normal manner both as regards mechanical efficiency and nervous control. The fact that Beddard, Pembrey, and Spriggs gave preference to the indirect alveolar-air method, despite its possible inaccuracies, may be attributed to the lack of a satisfactory technic for blood analysis.

Van Slyke's Method for Determining the Bicarbonate Content of the Blood.

During the present year a direct and exact method has been introduced by Van Slyke[4] for determining the bicarbonate content of the blood as measured by the power of the blood plasma to combine with carbon dioxide. This method not only eliminates the error of measuring the blood alkalinity by an indirect method but has the added advantages of simplicity, rapidity, and accuracy. It avoids, furthermore, the error due to personal factors. Van Slyke's apparatus consists of a glass pipette in which the plasma is acidified and the bound carbon dioxide is set free. The volume of gas may be read directly from the scale on the instrument or may be expressed in terms of

[2] Lancet, 1903, i, 1366.
[3] Jour. Physiol., 1913, xlviii, x.
[4] Method to be published in the Journal of Biological Chemistry in the near future.

volume per cent. carbon dioxide in the blood by reference to tables with corrections for temperature and barometric pressure. By a further simple calculation the figure obtained may be translated into terms of alveolar carbon dioxide tension.

This method affords a simple clinical means for determining the bicarbonate content of the blood. The blood estimation in conjunction with urinary findings gives an index of the production and elimination of acid bodies, a knowledge of both of which is essential to an interpretation of the clinical picture. In those instances in which an analysis of the blood cannot be made, valuable information may be obtained by determinations of the alveolar air.

Fridericia's Method for Determining Alveolar Carbon Dioxide.

In 1914 Fridericia[5] published a modification of an already well-known method for analysis of the alveolar carbon dioxide so simple and so easily applied to clinical use that it merits a brief description. A glass tube with stop-cocks is so arranged that 100 c.c. of expired air can be readily collected for analysis. The sample of air is shaken with an alkaline solution within the glass chamber and all the carbonic acid is absorbed by the alkali so that the amount of gas absorbed may be read directly in percentage from a scale marked on the tube.

Symptoms of Acidosis.

The symptoms due to acidosis vary. They range from the mere irritability, mental torpor, and lessened physical alertness of chronic low-grade acidosis through the hyperpnea, drowsiness, and headache of high-grade intoxication to the nausea and vomiting which presage the onset of diabetic coma. A careful study of the manner in which 22 cases have behaved while fasting has suggested that they may be divided into groups of four definite types. Any given case may react differently to fasting on different occasions. This classification is based on the study of acid production and elimination as determined by blood bicarbonate content and urinary acid excretion.

Group I is composed of cases which maintain a normal bicarbonate reserve of the blood throughout the course of their treatment.

[5] Berl. klin. Wchnschr., 1914, ii, 1268.

In Group II are placed the cases which recover, while fasting, from acid intoxication, sometimes so severe as to verge on coma.

Group III consists of those cases which show a low-grade acidosis as indicated by a subnormal bicarbonate reserve and heightened ammonia excretion.

Group IV includes those cases which develop acidosis while fasting, though previously no evidence of acid intoxication existed.

I. The cases which show no diminution of bicarbonate in the blood during the fasting period are milder diabetics. They are made aglycosuric easily by a few days of fasting and they do not develop untoward symptoms or clinical signs of acidosis during the fasting period. Their carbohydrate tolerance is relatively high and they have the ability to metabolize mixed diets of moderately high caloric value without showing glycosuria. The therapeutic indication in these cases consists in the adaptation of a diet to their tolerance in order that the aglycosuric state may be permanently maintained. (Chart I.)

II. Group II comprises cases which during the fast, recover from an acid intoxication which sometimes is so extreme as to verge on coma. These cases are frequently acute in course and develop symptoms of coma with alarming rapidity. There are generally a high glycosuria, a very heavy ketonuria, and a low sodium bicarbonate reserve in the blood (often less than one-half normal). The symptoms in these cases are anorexia, sluggish mental and physical reaction, beginning hyperpnea, and more or less extreme drowsiness. Fasting is followed by amelioration of all these symptoms. The sodium bicarbonate reserve in the blood rises and generally becomes normal on the third or fourth day, although the urine may not show a lessening in its reaction for ketone bodies until later in the treatment. In these cases the fast should be continued until aglycosuria results. (Chart II.) The patients are then treated by the method in use in this hospital[6] until they remain permanently aglycosuric.

III. The cases which show a low-grade chronic acidosis before, during, and after their fast comprise the most difficult group to treat successfully. (Chart III.) One may suspect that a patient belongs in this group from some or all of the following signs:

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[6] The details of the general and dietetic management employed will be described in a paper to be published later.

1. Chronicity of the diabetic symptoms.
2. Lessened mental acumen.
3. Lessened physical alertness.
4. Tendency to a low-grade but continuous glycosuria.

CHART I.—Patient, aged thirty years. Aglycosuric during the period covered by this chart. The power of the blood plasma for combining with carbon dioxide fell below normal only during the period of adjustment following a high carbohydrate diet. The ammonia curve was normal throughout.

5. Low carbohydrate tolerance.
6. Acertain degree of obesity.
7. Persistent lipemia.
8. Lessened sodium bicarbonate reserve in the blood.
9. A slight but extremely obstinate ketonuria.

These characteristics, as well as the readiness with which the acidosis is increased by fat feeding, suggest that the faulty metabolism of carbohydrate is complicated by difficulty in the complete combustion of fats.   The fasting period required to clear up glycosuria may be long.   During this period the urine may not become negative in its reaction for ketone bodies, and the bicarbonate content of the blood
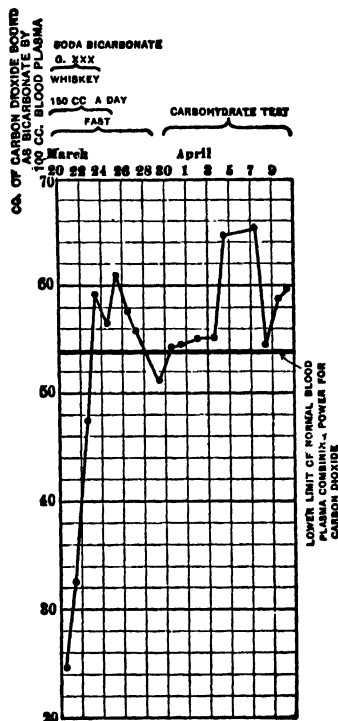
CHART II.—Patient, aged seventeen years.   High glycosuria and ketonuria from March 19 to 26.   The power of the blood plasma for combining with carbon dioxide rose to normal during the fast, and remained normal after.

may maintain its subnormal level.   When the aglycosuric condition is reached the patient may still show a low carbohydrate tolerance, rarely more than 60 to 100 grams of carbohydrate in the form of green vegetables alone, and may tolerate only from 0 to 15 grams when allowed a mixed diet of low caloric value (15 to 25 calories per kilogram of body weight).

An excessive supply of fat, even of body fat, together with a low carbohydrate tolerance, apparently plays an important part in the symptomatology of patients in this group, and it is toward the correction of these conditions that treatment should be directed. A continuous subcaloric diet with fast days interpolated twice a week
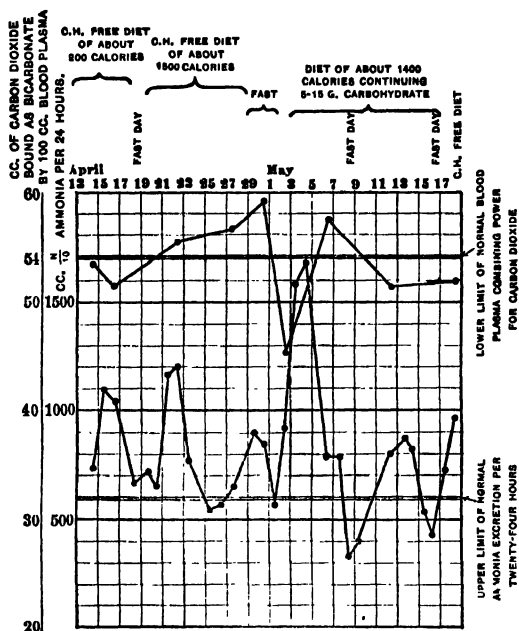
CHART III.—Patient, aged eighteen years. The patient was observed for fifteen months. This chart is taken from the middle of this period. During the first months of treatment the degree of acidosis was continually higher. At this period and thereafter the acidosis was less marked. Prolonged fasting caused a slight decrease of the power of her blood plasma to combine with carbon dioxide. This increase in the acidosis is not characteristic for the group but is a reaction which may be expected in fasting persons. The ammonia excretion, always high, increased still further to meet the demand for regulation. (These phenomena will be published in detail in another paper.)

rapidly rids the patient of excessive body fat, while the lowered total caloric intake allows of a greater amount of carbohydrate in the diet without the appearance of glycosuria. Even though the loss of weight may appear excessive it is not to be feared. In some instances as much as one-fifth of the body weight has been sacrificed before the

clinical, blood, and urinary evidences of acid intoxication have disappeared. Lipemia generally disappears rapidly with the loss of body fat. The patient becomes tolerant of more carbohydrate, the urine loses its reaction for ketone bodies, and the blood gradually
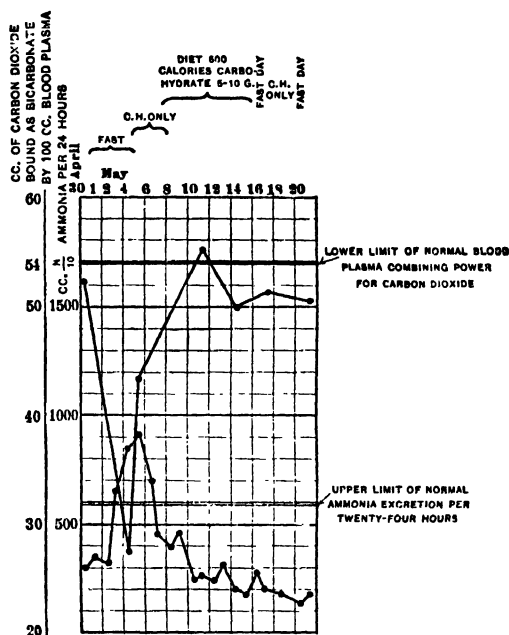
CHART IV.—Patient, aged twelve years. On admission she had a slight glycosuria and slight ketonuria. During the fast the ketonuria intensified while the glycosuria disappeared, and they both have been absent since. During the fast the degree of acidosis increased to a dangerous extent. The ammonia excretion, though increased, was inadequate. The fast was terminated and acidosis disappeared as measured by the power of the blood plasma to combine with carbon dioxide—the excretion of ammonia and ketone bodies. Two subsequent fasts were attended by no untoward return of these phenomena. (The lower normal level for the power of the blood to combine with carbon dioxide in a child, aged twelve years, should be placed between 50 or 52 instead of at 54.)

rises in its bicarbonate content. Not infrequently months of treatment are necessary to obtain good results in these patients. But persistence, no matter how discouraging treatment may appear, is almost always rewarded. So far it has been possible to discharge all of these cases on a diet sufficient for their comfort and usefulness

and no tendency to a spontaneous return of the former symptoms has been observed.

The cases comprising Group IV are those which develop signs and symptoms of acidosis during the fasting period, though previously no evidence of an acid intoxication may have been shown. (Chart IV.) The hidden danger of impending acidosis may be masked by their apparent state of good health and nutrition. Glycosuria sometimes appears to be the only abnormal factor in an otherwise satisfactory status. Urinary tests may show either a heavy or a negative reaction for ketone bodies. On the second or third day of the fast vague symptoms with irritability may appear. This condition is at first attributed to hunger, but on the following day the symptoms became intensified and nausea may develop or drowsiness supervene. Then the significance of the situation is recognizable. Should fasting be continued still longer, hyperpnea or vomiting, which usually indicate the onset of coma, may occur. During the development of these symptoms, even though the urine may sometimes gradually become clear of glycosuria and the ketone reaction remain slight or only mildly positive, the analysis of the bicarbonate of the blood shows an increasing tendency toward the danger zone. This group emphasizes the necessity of an early recognition of the onset of acidosis and illustrates the advantage of a clinical method which aids in its detection. In these instances acidosis may be manifested when fasting is instituted. The daily analyses of the blood indicate the degree of acidosis and provide the opportunity to control the dangers to which such patients are subjected during the fasting period. A progressive downward tendency of the bicarbonate content of the blood offers a clear-cut therapeutic indication. In these cases fasting should be discontinued. If such a patient is put on a low protein-fat diet the symptoms of acid intoxication may disappear after the first feeding and the bicarbonate content of the blood continues to rise until a normal level is reached and danger of coma is past. After several days of such diet a second fast has always, thus far, been well tolerated and has cleared up the glycosuria successfully.

Twenty-two diabetics have been studied with reference to the effect of prolonged fasting on acidosis. They may be divided into the groups just described, according to their response to fasting as follows:

Group.	Number of cases.	Percentage of cases.
I	10	45.4
II	2	9.1
III	8	36.4
IV	2	9.1
Total	22	100.0

The ten cases (45.4 per cent.) comprising Group I were benefited by the fasting method. They were rendered aglycosuric very rapidly and their stay in the hospital was very materially shortened.

The effect of fasting in the cases of Group II (9.1 per cent. of the series), which show evidence of being in a state of profound acid intoxication, is especially striking. These cases improve, within twenty-four hours, in such a manner that they may be considered out of danger of coma, although they were admitted to the hospital bordering on this state. The two cases cited received sodium bicarbonate (30 grams per diem until the blood bicarbonate reached normal) together with whisky (150 c.c. per diem divided into ten doses). Recently we have treated two additional cases in Group II; they fasted without receiving either bicarbonate of soda or whisky and improved in an equally satisfactory manner. The radical departure in this from previous methods consists in not giving carbohydrate to ward off coma. Certain of our results show that the feeding of carbohydrate in such cases may actually precipitate coma.

The patients comprising Group III (36.4 per cent. of the cases studied) were freed from a chronic low-grade acidosis by prolonged fasting and subsequent continued subcaloric feeding. The temporary undernutrition is not harmful, and temporary loss which is encountered is rapidly made good by improvement. Without the temporary delay, subsequent improvement is often unattainable.

The remaining cases—Group IV—(9.1 per cent. of the series) are those in which serious or fatal results are possible from prolonged fasting if the fasting is not controlled and suitable warning is not heeded. The clinical symptoms alone may not afford sufficient indication of the threatening danger, but the use of the methods described renders fasting safe. Under suitable control, therefore, the fasting treatment can be conducted with safety even in cases of this type.

SUMMARY.

The following conclusions may be briefly summarized thus:

I. The object of the fasting treatment of diabetes mellitus is to render the patient permanently free not only from glycosuria but also from acidosis.

II. The term acidosis signifies not an actual acid reaction, but an accumulation of acid bodies in the blood and tissues sufficient to neutralize enough of the bicarbonate there present to reduce the alkaline reserve below normal.

III. Urinary tests for acidosis are often misleading in that they are indicative merely of the excretion of acid bodies and are not a true index of their accumulation. An analysis of the blood bicarbonate as determined by the power of the plasma to combine with carbonic acid, on the other hand, offers an accurate measure of the accumulation of fixed acids and gives a true index of the degree of acidosis present.

IV. For convenience of description the diabetics above described were classified into four groups. This classification is based on the type of response to prolonged fasting as indicated by the degree of blood alkalinity.

V. When the degree of acidosis present is determined daily by the method described, experience so far indicates that all cases of diabetes may be treated by the fasting method safely and with benefit.

INVESTIGATIVE AND SCIENTIFIC PHASES OF THE DIABETIC QUESTION.

THEIR PROBABLE RELATIONS TO PRACTICAL PROBLEMS OF CLINICAL MEDICINE.*

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A discussion of diabetes at the present time must consist in statements more of problems than of conclusions. The attempt will be to treat the subject in its living and changing present state, with full realization of the risk of mistakes. I am indebted to the kindness of several investigators for permission to mention important unpublished work. In view of the magnitude of the subject, it is obvious that various important phases must be omitted altogether.

There is a fundamental question, What is diabetes? The word belongs historically to clinicians, and can rightly be applied only to the clinical disorder or its experimental counterpart in animals. The names "diabetes" and "glycosuria" are those of disease and symptom. The distinction is not a mere nicety or purism of speech, but is an actual requirement for clearness of thought and action. The majority of erroneous hypotheses would have been avoided if the makers had had in mind a clear distinction between glycosuria, which is a glucose in the urine, and diabetes, which is a defect of metabolism. The polyglandular doctrine would never have arisen except for the confusion between diabetes and some nondiabetic forms of glycosuria, especially that caused by epinephrin. Even totally depancreatized animals may by various injuries or intoxications be made free from both glycosuria and hyperglycemia, but they are still diabetic. Clinically, there are occasional cases of renal and other glycosuria which it would be incorrect to treat as diabetes. But the great preponderance of diabetes justifies the rule that any case of clinical

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* Delivered as the thirteenth Rush Lecture, Philadelphia, Jan. 21, 1916.

glycosuria should be considered diabetes unless it can be demonstrated to be something else.

To make the diagnosis of glycosuria merely because of the seeming harmlessness of the condition has proved to be a mistake. Slight glycosuria is nearly always mild diabetes. It yields to mild anti-diabetic treatment; but if neglected it may become associated with familiar diabetic complications, such as gangrene or acidosis, or it may pass more or less suddenly into the severe form. Though glycosuria is the traditional chief symptom of diabetes, it seems a justified therapeutic rule at present that every diabetic should be kept completely and continuously free from glycosuria. That physicians and patients should intelligently cooperate to this end in the stage of mild glycosuria is the most important requirement for reducing the death rate from diabetes.

When we return to the question, What is diabetes? it is possible at the present time to phrase a reply, with increasing agreement on the part of investigators, that diabetes is deficiency of the internal secretion of the pancreas. The history of this doctrine goes back to the early English and French clinicians who observed changes, especially atrophy, of the pancreas in some cases of diabetes. Brunner had tried in 1686 to remove the pancreas, and almost exactly 200 years later, after Claude Bernard and others had failed, von Mering and Minkowski succeeded. There followed a period of enthusiasm and dispute. A series of investigators published statements that they had removed the entire pancreas and found diabetes absent or transitory. Minkowski and his followers were able to retort that these authors had left small shreds of pancreatic tissue, and that the uniform production of severe diabetes was obtainable only by complete removal of the pancreas. Minkowski saw a few instances of mild or irregular diabetes when very small fragments were left, and severe diabetes when such fragments underwent necrosis. Sand-meyer left larger fragments, with the duct and part of the blood supply ligated, and saw that after marked atrophy of the pancreas remnant, requiring as long as thirteen months in one case, chronic diabetes and emaciation. resulted. This form may be compared with the unusual human cases resulting from pancreatic atrophy. An important matter at that time was the proof that diabetes is not due to

lack of the external pancreatic secretion; therefore pancreas remnants were left isolated from duct communication with the bowel.

The enthusiasm aroused by von Mering and Minkowski's discovery, the resulting belief in the pancreatic origin of diabetes, and the hopes for a new therapeutic era gradually died down in the face of baffling discouragements. These were essentially, first, the complete disappointment from pancreatic organotherapy, second, the repeated failures to find pancreatic lesions in diabetic necropsies, and third, the differences that were more and more insisted on between human diabetes and the form following total pancreatectomy in dogs. The result was a period when few persons were willing to affirm that diabetes is regularly and fundamentally a pancreatic disorder. Back in 1892, de Renzi and Reale reported a permanent and fatal diabetes in a dog, when a considerable fragment of pancreas had been left in its normal position and did not atrophy. The same authors made other claims which were erroneous, and neither they nor Pflüger gained sufficient insight into the matter to reproduce this form of diabetes. After many years, an idea suggested itself that the most promising way to study weakness of the pancreatic function is not found in the abrupt removal of the whole organ or in the atrophy of an isolated remnant. A more accurate and logical attempt to reproduce human diabetes will consist in removing portions of pancreatic tissue, leaving the remainder with its normal blood supply and its normal relation to the intestine. Accordingly, the above-mentioned form of diabetes was rediscovered in four different places at about the same time, the investigators all being ignorant of one another's work and of that of de Renzi and Reale.

The first in point of publication were Thiroloix and Jacob, who made two important observations: first, that when a sufficient portion of a dog's pancreas is removed, leaving the remainder in its normal connections with duct and blood vessels, this remnant retains normal appearance and supplies enough pancreatic juice to maintain the animal's nutrition, yet diabetes develops and runs a gradual downward course much like the human disease; second, in suitably prepared animals, this downward progress can be produced or hastened by overfeeding with carbohydrate. These authors made several mistakes, among them the statement that the islands of Langerhans

are unchanged; but a 1913 announcement by Thiroloix and More indicates that they have seen the changes and propose to study them, being still ignorant, like most Europeans, that this has already been done in America.

The second in point of publication was Helly, who studied only the glycogen, finding that in this form of diabetes the animals retain more or less glycogen, here resembling human diabetics.

The third to be published was my own work, which dealt with various points concerning this type of diabetes, compared it with the normal and with other forms of diabetes and of glycosuria, and also, thanks to the cooperation of Prof. F. B. Mallory, described for the first time the specific changes in the islands of Langerhans in experimental diabetes—inasmuch as one previous statement by Minkowski concerned an isolated and accidental observation, and from his phrase "shrunken and altered islet cells" it is not possible to say definitely whether he saw the late stages of hydropic degeneration or other changes that may occur in a subcutaneous pancreatic graft.

The fourth in point of publication was Homans, who discovered this form of diabetes in cats and made the most accurate histologic studies, as will be mentioned later.

Experimental Imitation of Human Diabetes.

Now the question is proper, In what respects can human diabetes be imitated by this method in experimental animals?

1. The onset may be ·similar. When nine tenths or more of the pancreas is removed, the onset may be acute. When seven eighths or sometimes less of the pancreas is removed, the onset may be gradual, with longer or shorter periods of slight or intermittent glycosuria.

2. The effect of carbohydrate food is similar. When the work of Thiroloix and Jacob is repeated with adequate controls, it can be proved that dogs after suitable operations can be kept for months and apparently for years in the best of health on a diet within their tolerance; but a diet in excess of the tolerance gives rise to a permanent and fatal diabetes. Sugar càn produce diabetes in some animals, which, at least for a considerable time, can live on starch safely.

3. Individual idiosyncrasies are somewhat similar, as mentioned

in Homans' recent paper. It is curious that some human patients go downhill so readily and lose tolerance so rapidly under improper diet, while others can keep up glycosuria for months and perhaps years with surprisingly little damage to either health or tolerance. Conditions in animals are more uniform and controllable; but yet, after apparently the same sort of operation, animals show variable hypertrophy of the pancreas remnant; and, aside from this, some animals will endure weeks or months of diet beyond their tolerance before they pass into hopeless diabetes, whereas other animals within a few days develop an intensity of diabetes which nothing can check.

4. The diminished permeability of the kidney for sugar is similar. When glycosuria is stopped, the blood sugar may remain above normal for indefinite periods, just as in human patients. Also, animals which have been kept free from glycosuria may show the blood sugar increasing steadily through weeks and months, with the renal impermeability apparently keeping pace, before glycosuria finally begins, so that the simple excess of sugar in the blood seems sufficient to produce sugar impermeability.

5. Nervous influences may be similar. Physicians have sometimes said, "This case is not diabetes; it is merely nervous glycosuria." A partially depancreatized dog may be subject to such nervous glycosuria, so that merely a trip to the operating room gives rise to a little sugar in the urine. This is true especially in small, nervous dogs; it has not been noticed in big, phlegmatic dogs. I have not yet seen an instance of permanent diabetes of psychic origin in any animal. This might be possible, but there is difficulty in producing in animals any such prolonged and intense nerve strain as human beings sometimes endure.

6. Traumatic diabetes occurs in occasional predisposed animals, just as in predisposed human subjects. Two dogs showed beginning or aggravation of diabetes after brief anesthesia for simple subcutaneous operations. One of these could not be made sugar-free by fasting, the condition being thus analogous to a human case recently described by Joslin. A partially depancreatized cat, injured in an accidental encounter with a dog, showed no glycosuria at the time; but as the appetite gradually returned, severe diabetes suddenly began, two weeks after the accident, and continued until checked by

fasting. The many months of observation on fixed diet, before and after the accident, make it probable that this was a case of delayed onset of diabetes after traumation, exactly as recorded in various human patients. As traumatic diabetes is unusual among patients, so also these three are the only ones seen among hundreds of animal observations.

7. Diabetes decipiens is an old name for glycosuria without polyuria. It means deceiving or deceptive diabetes, and originated from the fact that early clinicians sometimes failed to diagnose diabetes when there was no polyuria. Probably such a condition means always one of two things, either an organic or functional abnormality of the kidney with diabetes, or a mild type of diabetes. Severely diabetic animals show the diuretic effect of carbohydrate like typical human diabetics, but it is also possible to have animals temporarily at a stage at which doses of sugar or starch produce marked hyperglycemia and glycosuria without much change in diuresis, or with an actual diminution of urine, thus indicating that the mere percentage of sugar in blood or urine is not the decisive factor.

8. These animals do not show such cachexia and impaired wound healing as totally depancreatized animals, but they go through operations successfully, as human diabetics usually do under suitable conditions. Severe diabetes in animals runs its course in a few months, and this fact may in some degree explain the relative absence of complications. In the later cachectic stages, there is increased tendency to sore eyes, distemper, skin troubles, cystitis, and what in human patients might be called a form of gangrene; that is, insignificant injury or pressure results in superficial infections and ulcers; these slowly extend instead of healing. There has never been any evidence for the belief that diabetic complications are due to high blood sugar. Against this belief is the fact that normal animals receiving sugar injections for long periods show no such complications, and this type of diabetic animals may have very high blood sugar for much longer periods than totally depancreatized animals, yet their wounds heal better. Diabetes is a specific disorder of nutrition, and it diminishes the ability of all the body cells to resist infection, repair injury, or withstand the ordinary wear and tear. Such a conception gives a rational explanation of the increased susceptibility of the

human diabetic to cataract, retinitis, neuritis, arteriosclerosis, pyogenic infections and tuberculosis, and the improvement of these conditions when proper therapy improves cellular nutrition; while the diabetic dog shows increased tendency not so much to these identical complications as to the corresponding canine afflictions.

Differences between Clinical and Experimental Diabetes.

We now come to the chief points of alleged difference between clinical and experimental diabetes. First is the dextrose-nitrogen ratio, which is Lusk's figure of 3.65 in the severest human diabetes, whereas removal of the dog's entire pancreas gives only the Minkowski ratio of 2.8. Eppinger, Falta and Rudinger reported that they obtained ratios above 2.8 by giving epinephrin to a depancreatized dog, or by removal of three parathyroids in addition to the pancreas; but such a statement, in the sense of anything more than a temporary flushing out of preformed carbohydrate, is open to serious question and cannot be accepted unless confirmed. Phloridzin gives a 3.65 ratio in man and the dog, but only a 2.8 ratio in the cat, rabbit and goat. Here the cause of glycosuria, phloridzin, is the same, but the ratio is different because of the difference in species. Total removal of the human pancreas should theoretically give a ratio of 3.65; but it is not permissible to say that human diabetes is not pancreatic because pancreatectomy gives only a 2.8 ratio in the dog. This is one of the reasons why I am engaged in producing diabetes in various species of animals, and it will not be surprising if one or more species may be found in which pancreatectomy gives a 3.65 ratio.

The second point of difficulty has been the great increase of total metabolism in the totally depancreatized dog. The answer here is twofold. First, although the partially depancreatized animals have unfortunately never yet been studied in the respiration calorimeter, the available observations concerning the food and urine indicate that these animals will be found to resemble human diabetics rather than totally depancreatized animals. Second, although the increase of metabolism in severe human diabetes reported by Benedict and Joslin was disputed by other authorities, now, on the basis of some recent observations already discussed by Geyelin and Lusk

in a meeting at the Academy of Medicine in New York, it is agreed that some cases of human diabetes may show a well-marked increase of metabolism in comparison with nondiabetics under similar conditions. A striking example is the patient of Geyelin and Du Bois, who after five days of fasting still had a daily nitrogen excretion in the neighborhood of 30 gm. and a total metabolism at least 3 per cent. above normal. There may still be discussions concerning the frequency or the cause of such increased metabolism, but the existence of such human cases and the similarity to diabetic animals is established.

The third point is the difficult one of anatomic changes in the diabetic pancreas. Using tissues stained by the ordinary methods in Mallory's laboratory, I observed that the cells of the islands of Langerhans in dogs with early diabetes begin to show vacuolation; and as the diabetes progresses, this vacuolation increases, the nuclei become pyknotic, and cells and islands finally disappear. Further work has confirmed the impression that the changes are strictly specific to diabetes, they are not due to operative trauma or other extraneous causes, and they can be produced at will by feeding predisposed animals in excess of their tolerance, the clinical and anatomic changes running parallel.

Weichselbaum had described changes of this sort in the diabetic human pancreas, under the name of hydropic degeneration, and he asserted that he had found this or other lesions of the islands in all of his 186 diabetic necropsies. Other investigators—the latest being Major—have not been convinced of the existence of any specific alterations in the diabetic human pancreas. No pathologist has ventured to claim that he could go through the necropsy material of a hospital and pick out the diabetic from the nondiabetic cases, and unless a test of this sort can succeed in at least a fair proportion of cases, we have no microscopic diagnosis of diabetes.

One statement can be made positively: The typical diabetic human pancreas does not show the striking and generalized alterations of the islands present in experimental animals, for if it did, no conscientious pathologist could miss them. It is not justifiable to fall back on difference of species as the whole explanation. With minor variations, the diabetic degeneration of the islands has been found similar in several species of diabetic animals, including the monkey. I was

privileged to see material obtained by Dr. Alonzo Taylor from a case of diabetes following pancreatic operation in a human patient; the lack of perfect fixation, unavoidable because of the circumstances of the necropsy, may make judgment difficult. Ghon and Roman described a case of youthful diabetes with very small pancreas due to developmental defect, and the changes in the islands of Langerhans were obviously identical with those in partially depancreatized animals.

The classical work in the microscopic study of experimental diabetes is that of Homans. Lane and Bensley had demonstrated that the cells of normal islands are of two distinct types, a less numerous alpha type and a more numerous beta type, distinguishable by the differential staining of their granules. Whereas ordinary stains, as mentioned above, make it appear that the entire islands degenerate in experimental animals, Homans proved by the special granule stains that only the more numerous beta cells show the exhaustion and degeneration, while the less numerous alpha cells remain intact. The easiest supposition is that the specific secretion of the beta cells is concerned in carbohydrate metabolism. The function of the alpha cells is entirely unknown. In a dog with spontaneous diabetes studied by Dr. Krumbhaar, the pancreas was about twice as large as normal, and degeneration was seemingly present in both beta and alpha cells. Homans points out that in cats the changes in all the beta cells are practically simultaneous, whereas in dogs with early diabetes occasional cells show exhaustion while the majority still appear normal. Homans has described a similar occurrence in human diabetes, namely, that although much of the island tissue may appear normal, yet some beta cells in some islands show typical hydropic degeneration. It would seem that the most promising prospect for the microscopic investigation of diabetes lies in this direction, where Homans has led.

The functional element in human diabetes must also be considered, and is emphasized by the results of treatment. When the beta cells of a dog are all degenerated, nothing can stop the diabetes. But human cases of the severest type can be checked in a few days, and within a few days more some of them show a rather surprising carbohydrate tolerance. By permission it is also possible to mention a patient of Dr. Eugene Du Bois whose urine easily became sugar-

free; then, on overfeeding with carbohydrate-free diet, there was an abrupt development of total diabetes as demonstrated by the D/N ratio and the respiratory quotient, and then a quick clearing up of the glycosuria on fasting—all within a very few days. These phenomena are suggestive of functional rather than anatomic alterations.

Opie founded the insular hypothesis of diabetes on clinical-pathologic observations, and the experimental evidence for it is good, inasmuch as Macallum, Laguesse and others proved the absence of diabetes when apparently nothing is left of the pancreas except island tissue, and the recent experiments show the presence of diabetes when apparently nothing is lost except the beta cells of the islands. It is not strange if there are some discrepancies between a spontaneous disease and the consequences of surgical reduction of an organ. We properly speak of human diabetes as pancreatic, because of evidence that deficiency of the internal pancreatic function is the central feature. But the cause back of the deficient function may be a disorder of the abdominal nerves, and this cause may to greater or less degree disturb the function of other organs than the pancreas. Large wet kidneys, and sometimes a hyperemic liver, are traditional in diabetes, with no definite pathologic changes in either organ. The recent treatment has brought into prominence the fact that although the kidneys are microscopically normal, abnormal renal function is the rule rather than the exception in severe diabetes. This is evidenced especially by the marked tendency to edema, which is shown to be associated with salt retention. Urea excretion is generally above normal (McLean). Decision concerning the incidence and significance of organic changes in the pancreas obviously requires further study by the best staining methods, and there is a real dearth of suitable necropsy material. Pancreatic specimens should be taken fresh, within an hour of death if possible, and should be cut almost as small as curettings, that is, a very few millimeters in dimensions, to permit quick penetration of the fixative. A simple fixative for the purpose is Zenker fluid without acetic, or with only 1 per cent. acetic acid. Physicians who can obtain such material would render a service by sending it to some one interested in the study.

The fourth and last of the differences to be considered between clinical and experimental diabetes is ketonuria or acidosis. It is an

old statement that the diabetic patient dies in coma and the diabetic animal dies in cachexia. Totally depancreatized dogs may show ketonuria, generally slight, and a few authors have reported the death of some such animals in a comalike state. But the cachexia is so pronounced that clinical judgment may be difficult, and Sassa found no reduction in the alkalinity of the blood of depancreatized dogs. Fasting phloridzinized dogs show more or less ketonuria, and coma-like conditions have been described, but there is possible confusion due to cachexia or phloridzin intoxication, and, although blood tests are in progress, it is too early to report as yet concerning the possibilities of acidosis. It is fully understood that dogs have much less tendency to ketonuria than man, and that is one of the reasons for the present attempts to produce diabetes in species that are more like the human being in this respect. But there are two answers to the traditional argument, even when dogs serve for the comparison. First, totally depancreatized dogs and even Sandmeyer dogs have poor fat-absorbing power, whereas human diabetics have been stuffed with fat; and when the customary overfeeding of human diabetics is stopped, death in coma will be less frequent. Second, under suitable conditions, even dogs seem susceptible to coma. I have seen three dogs with fatal intoxication, and either in dogs or other species, there is some hope for an adequate imitation of human diabetes in this respect.

Diabetic Acidosis.

Here it may be well to introduce a few remarks touching the present status of the subject of the so-called diabetic acidosis. Until recently the doctrine seemed comfortably established that ketonuria is the result of lack of carbohydrate, though von Noorden insisted on the influence of individual idiosyncrasy and habituation, and Magnus-Levy admitted that some unknown factor might sometimes play an accessory rôle. It was also universally believed that the one therapeutic measure of preeminently proved necessity in diabetes was the neutralization of acid intoxication by large doses of alkali. The occasional fast-days or vegetable days recommended by Bouchardat, Cantani and Naunyn were known to diminish acidosis, but there exists sufficient evidence that prolonged fasting was generally

considered inadvisable or even dangerous in severe diabetes. Guelpa's practice of fasting is not an exception, for his cases were not severe. Almost two years ago, on the basis especially of the observed benefit of fasting in diabetic dogs, I tried prolonged fasting in severe human diabetes, and found that not only did the glycosuria cease, but also there was practically complete cessation of acidosis, as judged by the clinical symptoms, ketonuria, ammonia excretion, and the dosage of bicarbonate needed to make the urine alkaline. Since that time the study of the acidosis has been more extensively and accurately carried out by Drs. Van Slyke, Cullen and Stillman, who have used alveolar air and hydrogen ion concentration methods, but especially the new Van Slyke method for determining the carbon dioxid capacity or sodium bicarbonate concentration of the blood. It is desired here only to mention briefly some results of the fasting treatment and their bearing on current theories of acidosis.

First, consider the theory of acidosis due to lack of carbohydrate. Fasting produces a slight ketonuria in normal persons. When it is followed by carbohydrate-free diet, a marked acidosis with typical clinical symptoms may result, as shown especially by Landergren and Forssner. But when the typical severe diabetic fasts, symptoms of coma promptly clear up, the ketone and ammonia excretion fall precipitously, the carbon dioxid of the alveolar air and blood rises to an approximately normal level, and these things then remain so on strictest carbohydrate-free diet, sometimes even if the diet is heavy with fat; the ferric chlorid reaction remains negative, and there are only traces of acetone, a slightly high ammonia, and a slightly low blood alkalinity.

Then, there is von Noorden's theory of habituation; the severe diabetic becomes accustomed to living without carbohydrate, and therefore he develops the power of disposing of such a diet without ketonuria. But a patient recently treated by Dr. Stillman was a child aged 26 months, who had had diabetes only three weeks and had constantly had carbohydrate in the diet, and when placed suddenly on complete fasting the child's acidosis cleared up promptly and completely. On the other hand, patients who have had moderate diabetes and have lived on carbohydrate-free diet for months or years may show an actual increase of acidosis when they fast.

Then there is the other von Noorden theory of individual idiosyncrasy as respects the function of burning acetone bodies; some diabetics have a specific weakness of this function, so that they cannot be made free from acidosis and are in imminent danger of coma. It will be noticed that the present therapy turns the former question directly around; these patients with the highest acidosis are the very ones who most often clear up completely under the conditions in which normal persons or milder diabetics develop acidosis; and concerning the very patients who were previously suspected of an abnormally low power of burning acetone bodies, we must now ask, Have they an abnormally high power of burning acetone bodies?

The glycogen supply has been held important; Forssner, for example, believed that by fasting and exercise to diminish the glycogen, he disposed normal persons to increased acidosis. Also, diabetics with acidosis have been known to go into coma after overexertion. But the normal person and the mild diabetic who develop acidosis on fasting are certainly richer in glycogen than the severe diabetic, whose acidosis clears up on fasting. Moreover, the analyses in the literature indicate that patients who die in coma may possess considerable glycogen. Von Noorden gives the record of two patients who received levulose and had 2.5 per cent. liver-glycogen at necropsy, yet they died in coma. Though exercise should be used judiciously, and should not be commenced until a threatening acidosis has been reduced, yet, after this time, many severe diabetics are benefited by vigorous exercise during fasting or on carbohydrate-free diet thereafter; the result is not ketonuria or coma.

The burning of sugar has been deemed all-important, and it might be considered that diabetic acidosis clears up on fasting because the glycosuria stops and the patient now burns his own protein sugar, whereas previously he was losing more or less protein sugar. But in the above-mentioned severe case of Geyelin and Du Bois, the acidosis dropped enormously on fasting even while the dextrose-nitrogen ratio was still 3.65 : 1. Benedict and Osterberg found that the ketonuria of phloridzinized dogs fell from 50 to 90 per cent. when they were fed protein, though all protein sugar was quantitatively excreted. Carbohydrate, frequently in the form of bread and milk, has been widely used in attempts to ward off coma. A 26-months-old diabetic baby was

mentioned previously. By mistake, toward the close of his fasting period, this child received a meal of bread and milk. The exact quantity is not known; but the child never had total diabetes, and the urine had become almost sugar-free, so it is fair to assume that some carbohydrate was burned. But the result was a sudden heavy glycosuria and ketonuria, an abrupt fall in the blood carbon dioxid, and a return of threatening acidosis symptoms; and all these cleared up on continuance of fasting.

When the severe diabetic fasts, there is generally a marked fall in metabolism, and this might be assumed as a basis for a diminished production of acetone bodies. But in the Geyelin-Du Bois case the acidosis dropped while the metabolism was still high. Children are supposed to be specially subject to acidosis, but children with severe diabetes clear up on fasting without difficulty. The acetone bodies are derived chiefly from fat, and Folin and Denis are the latest to describe the tendency of fat persons to acidosis while fasting, so it might be thought that the severe diabetic loses his acidosis readily because he is so emaciated; having so little fat to burn, he must burn something else instead. But the baby mentioned above was as plump and chubby as could be wished, and the acidosis cleared up. On the other hand, a man with only slight acidosis but most extreme emaciation failed to become free from acidosis on fasting, and his ketonuria stubbornly resisted the subsequent treatment for a considerable time. Also, Dr. Geyelin has made the observation that one or two of his patients with moderate diabetes showed slight persistent ketonuria as long as they were undernourished, but when the calories were brought up to the requirement by merely increasing the carbohydrate-free diet, the ketonuria ceased.

The state of intoxication here discussed is familiar to everybody and has become an old story in diabetes; but what is this condition, and what name can properly be applied to it? The name "acidosis" sounds most scientific at the present time. The school of Naunyn and Magnus-Levy has insisted on the diminished alkalinity of the body fluids as the essential feature, and on this basis recent work has brought the dyspnea and coma of diabetic acidosis into relation with the similar symptoms in uremia and other intoxications, including some disorders of childhood. It is true that these diabetics when

left to themselves regularly show an excretion of abnormal acids in the urine, and a diminished alkalinity of the blood. But the one outstanding historical feature is the failure of the alkali therapy for coma. The excuse about not being able to give enough alkali to neutralize the enormous quantities of acid present is open to some question. If one does succeed in giving enough bicarbonate to turn the urine alkaline, a diabetic on the verge of coma may not show the same perfect clearing of symptoms as an acid-poisoned rabbit. Take a patient who is just going to sleep, of the type which, as past experience has shown, in a majority of cases cannot be saved by alkali, and give him no alkali, merely let him alone without food; in twenty-four hours he may be out of danger. That does not look like such enormous quantities of acid accumulated in the fluids and cells. Von Noorden and a few others have insisted that some diabetics die in coma when the urine has been kept constantly alkaline for a considerable period and up to the end. I have personal knowledge of two patients who died when the alkalinity of the blood had been kept normal or above normal by means of bicarbonate. Dr. Joslin in Boston seldom if ever uses bicarbonate now. Sodium bicarbonate promotes diuresis under favorable conditions, and the intravenous infusions raise blood pressure. But there is the old instance in which Hilton-Fagge obtained a similar brief reviving effect by the use of an acid solution intravenously. Last month Poulton published a brief report, and by comparison of his figures with those of Barcroft, he draws the conclusion that, according to the curve of hemoglobin dissociation, a normal person after moderate exercise has a more acid blood than a diabetic in coma. The carbon dioxid findings appear not to agree with this.

Other possible names for the intoxication are ketogenesis, ketonuria and ketonemia. Against ketogenesis it may be objected that more or less production of one or more acetone bodies is a regular process of normal metabolism. Ketonuria lays stress on the increased concentration of acetone bodies in the urine, rather than in the blood and tissues, whereas the latter is the more important factor, and is supposed to be especially dangerous in some cases of retention with very little ketonuria. Ketonemia correctly designates the circulating excess of acetone bodies, but, if used to cover the entire condition, it

implies that specific poisoning with acetone bodies is the essential feature. Ehrmann and collaborators supported this view by producing an alleged imitation of diabetic coma in young dogs by feeding or injection of the alkali salts of butyric or diacetic acid.

According to this view the body burns and excretes all the acetone substances it can, and the excretion is facilitated by alkali; but owing to the excess present, these defenses are inadequate and the body is poisoned, even though the substances are present not as free acids but as neutral salts. But in Ehrmann's experiments, ketonuria was slight; and it remains unexplained why the normal organism overwhelmed with the alkali salts of keto-acids fails to excrete them in the large quantities characteristic of the intoxicated diabetic patient.

The toxicity of acetone bodies is relatively slight, so that large quantities of them must be present for fatal poisoning. In diabetic necropsies Sassa has lately found values somewhat lower than those reported by Magnus-Levy. When patients with only slight ketonuria go into coma, the assumption has been that the acetone bodies were retained. Weiland claims to have found at necropsy no great increase of beta-oxybutyric acid in the organs of such patients; but he does not give figures. Marriott's tables show a severe diabetic not in coma with 65 mg. of total acetone bodies per hundred gm. of blood, whereas a child in coma had only 48 mg. per hundred gm. of blood. Clinicians consider a sudden high ketonuria especially dangerous, and in the Landergren-Forssner experiments the acute ketonuria was associated with decided symptoms of intoxication. Yet Benedict and Lewis produced by phloridzin in human subjects a dextrose-nitrogen ratio of 3.6, and a sudden heavy ketonuria with ammonia excretion above 4 gm. daily, and symptoms such as seen in diabetic patients were absent.

The name "diabetic coma" might appear safe and conservative; but the condition is not always diabetic, and there is a question whether it is always coma. Forssner was not a diabetic, yet he showed the typical intoxication, and might well have gone into coma if the experiment had been prolonged. Coma is merely an end-state, and there must be a name for the less severe degrees of the intoxication. The intoxication observed by Folin and Denis in fasting obese women

has been mentioned. The subjects need not be obese. Bönniger and Mohr observed ketonuria of over 24 gm. in a fasting woman; Gerhardt and Schlesinger 40 gm. daily in hysterical vomiting; von Noorden 48 gm. on the third day of fasting of a girl with gastric ulcer. None of the persons who showed symptoms were deprived of carbohydrate in anything resembling the exquisite manner of Benedict and Lewis' phloridzinized subjects, who were free from such symptoms. Howland and Marriott have lately reported ketonuria, acidosis and intoxication symptoms seemingly not explained by carbohydrate deficiency, and in at least one patient on full mixed diet. Clinicians are aware that occasional diabetic patients die not in coma, but in what has been called diabetic collapse or heart failure. It is not known whether this is an expression of the same intoxication that causes coma.

Also, consider again the phenomena of fasting. Two patients excrete sugar on carbohydrate-free diet. One has little ketonuria or acidosis; the other has much. Apparently, one of them can resist ketonuria or acidosis better, or has less tendency to it, than the other. Then they fast, and the one who seemed to have poor resistance becomes free from ketonuria and acidosis. The one who seemed to have good resistance does not become free. The ammonia in the latter's urine may or may not increase; the carbon dioxid of alveolar air and blood falls perhaps dangerously low, and there are intoxication symptoms. From 5 to 20 gm. of sodium bicarbonate may raise the carbon dioxid and clear the clinical symptoms. The questions arise: Why are there such marked signs of acidosis chemically and clinically, when such a small dosage of alkali suffices to remove them? Why does this patient, who appeared to resist acidosis so well, not form the small amount of ammonia required to neutralize his acids?

Then there are the rare patients who develop alarming symptoms on fasting. The cases may at first seem severe or only moderate. The glycosuria may clear up or remain heavy. But the acetone bodies and ammonia are high and the carbon dioxid low. Symptoms commonly associated with acidosis appear—weakness, malaise, nausea, vomiting. Maximum doses of bicarbonate may raise the alkalinity of the blood to normal, but do not save the patient. Consciousness

may be retained practically to the end. In an ordinary patient verging on coma, carbohydrate-free diet is a fairly sure means of producing coma. But in the type of patient here described, carbohydrate-free diet, given in time, may clear up the acidosis symptoms, and some days later a second fast is well borne and there is no further difficulty. The patient of Geyelin and Du Bois is the best therapeutic example of this kind of case to date. After five days of fasting, sugar, nitrogen, acetone bodies, and ammonia were high; intravenous bicarbonate infusions raised the carbon dioxid capacity of the blood to 30, but dyspnea and drowsiness gave unmistakable warnings. The patient was accordingly fed. His urine was as unfavorable as before; his carbon dioxid under smaller doses of alkali fell as low as 19, yet his clinical condition was transformed. A second fast cleared up everything successfully. A striking feature is found in the classical symptoms of acidosis present when the carbon dioxid reading was 30, and the marked clinical improvement in these symptoms when the reading had fallen to 19.

Further paradoxes and contradictions could be mentioned if space permitted. It is a sufficient summary to say that some empiric observations exist, but, fundamentally, nobody knows anything about acidosis. We are not even sure of a correct name for the diabetic intoxication. Yet methods are now available—methods for carbon dioxid and hydrogen ion concentration, and micro methods for acetone bodies. It is safe to say that diabetic intoxication and coma will now be studied more carefully and accurately, and within a few years our understanding will have advanced beyond the chaos here depicted. The condition may turn out to be a true acidosis, or a specific intoxication with acetone bodies, or varying combinations of the two elements; or it may be a poisoning with unsuspected intermediary products of fat metabolism. Even clinical observation should be sharpened. It is now probable that typical Kussmaul dyspnea or big breathing is characteristic of acidosis; yet Weiland mentions deep breathing with alkaline urine, and observations in various states of acidosis should easily show the facts. It is probable that patients do not show typical coma without acidosis, though they die with an obvious intoxication.

A further metabolic problem has been raised, since, in the severe

diabetics who clear up on fasting, Benedict and Joslin and Du Bois have found some higher respiratory quotients than seem explainable by combustion of the materials supposedly available. Strangely enough, there exists no satisfactory information concerning the amount of sugar present in the tissues under various conditions, but animal experiments now conducted by Dr. Palmer will throw some light on this subject. Joslin has suggested that the high quotients may represent combustion of acetone bodies. The lesson of clinical experience seems to be that, irrespective of individual idiosyncrasy, patients who have once experienced intense acidosis develop a peculiar power of disposing of these acid metabolic products. Perhaps it is a specific increased power of burning acetone bodies or avoiding their excessive formation; perhaps it is the same power of doing without carbohydrate, possessed by carnivorous animals. Again, as alkalies are known to increase the loss of acetone bodies, supposedly by facilitating their excretion, the simple explanation may be that the patient who has had high ketonuria has thereby developed a diminished renal permeability to acetone bodies, strictly comparable to the diminished permeability of the diabetic kidney for sugar, and that this raised threshold dams back the acetone bodies from the urine to greater extent than normal, so that they circulate in higher concentration, are more dangerous when there is great excess, but also under favorable conditions are burned in higher degree, according to the law that seems to govern all combustible substances. Dr. Fitz is at work on this and related problems. Altogether, the indications are that the treatment by fasting will result in throwing considerable new light on the theoretical conceptions of diabetic intoxication and acidosis.

In the practical therapy of acidosis, it is still a wise general rule to use alkali and other customary measures at the outset, as recommended in the first paper concerning the fasting treatment,[1] though under proper conditions and with adequate tests of the acidosis, nearly all cases can be managed without alkali. If alkali is used, the dosage can be smaller than formerly employed for threatened coma, and the injurious or dangerous effects of the large doses may thus be avoided. For the average patient, a proper fasting and dietary

[1] Allen, F. M.: Studies Concerning Diabetes, THE JOURNAL A.M.A., Sept. 12, 1914, p. 939.

régime quickly obviates the need for alkali. For the occasional patient who reacts badly to fasting, the practice in this hospital is not to resort to excessive doses of bicarbonate, but to terminate the fast by giving restricted diet. After a week or two of such diet, a second fast has regularly proved successful. In no case is alkali given for more than a very short period, and the ideal in all cases is to prevent the formation of the acid substances rather than to attempt to neutralize or eliminate them by means of sodium bicarbonate.

Treatment.

Leaving this digression concerning acidosis, let us return to our original question, What is diabetes? If it springs from deficiency of the internal secretion of the pancreas, what are the results or manifestations of this deficiency? The most obvious fault is in the sugar metabolism; here the distinctive feature of diabetes is not glucose in the urine, but diminished power to use glucose. But diabetes is probably something broader than this. The normal person eats food, digests it, and absorbs it into his blood, and his cells assimilate it for their nutrition. The diabetic eats, digests and absorbs normally, but does not assimilate normally. Diabetes may probably be defined as a specific deficiency of the power of assimilating food.

The question may be pushed further; what is the mechanism of this power, the reason for this deficiency? Concerning this, I have previously proposed a tentative hypothesis of "binding substances." The hypothesis is merely suggestive. It is entirely without chemical support. But since chemical methods have not yet been able to grasp the problem of the mechanism of diabetes, the negative chemical evidence is not decisive. In addition to the previous observations concerning the diuretic action of sugar, the following may possibly have some connection with this idea of the ability of cells to bind food substances. First, the accumulation of reserves, as when the body gains weight, diminishes tolerance and aggravates diabetes; whereas the reducing of the reserve stores, by low diet or by exercise, increases tolerance and checks diabetes. Second, any kind of food— except apparently alcohol, which has no storage form— weakens the assimilative power for other foods. Adding carbohydrate to a dia-

betic diet may give rise to ketonuria. Adding fat to a diabetic diet may give rise to glycosuria. This fact was partially obscured when it was the practice to give severe diabetics carbohydrate for the sake of relieving acidosis. Certainly an excess of carbohydrate, by forcing combustion of carbohydrate and also diminishing combustion of fat, may temporarily diminish ketonuria; but the injury to the patient's actual functional capacity is still evident. After a sufficient period of carbohydrate excess, there is greater ketonuria than before on the same diet as before; and, as shown above, it is difficult to maintain that ketonuria is due merely to lack of carbohydrate. The true conditions are more clearly observable under the fasting treatment.

The history of diabetic therapy, so far as it is significant or valuable, consists merely in an interweaving or alternation of two principles. One is restriction of the sugar-yielding elements of the diet, namely, carbohydrate and protein. The other is diminution of the total caloric value of the diet. The latter is the older. Willis reported benefit from a diet limited to milk and barley-water cooked with bread. There have been milk and vegetable cures since then; but whereas the authors aimed at a nontoxic diet, what they really achieved was undernutrition. Then came Rollo with his notion of animal food to strengthen digestion, and thus the benefit of carbohydrate restriction was accidentally discovered. The harmfulness of excessive protein-fat diet was soon recognized, and Bouchardat, Cantani and Naunyn introduced fast-days and lower diet. Then came the ritual of von Noorden's oat cure, and the widespread ideas concerning special properties in oatmeal, which ought not to have gained credence among those acquainted with the literature of previous carbohydrate and vegetable diets. Blum broke the spell by having the courage to declare that oatmeal is like any other form of starch; and then straightway others made the test, and it became established that there is no special virtue of any kind in oatmeal. The oat cure gave patients a few days' rest from protein-fat excess by substituting carbohydrate excess. Its advocates claimed that it avoided the undernutrition characteristic of other carbohydrate cures, by making up a diet adequate in protein and calories on the oat days; but the undernutrition was present in the fast-days or

vegetable days preceding and following the "cure," and therein lay the principal benefit. The oat cure merely shows that a large proportion of diabetics still manifest a surprisingly high carbohydrate tolerance when conditions are not too unfavorable. It is a failure in genuinely severe cases.

The fasting treatment has been previously outlined,[2] and details will be published later. The patients treated at the Rockefeller Hospital now number above sixty. It remains true that cases of diabetes of severest type have been made and kept free from glycosuria and ketonuria. There have been deaths from complications and accidents before treatment could be completed, and physicians will be able to judge concerning these when the details are published. No complication has appeared in any case after treatment, unless the one death from tuberculosis be classified here. Five patients broke their diet and are dead. In the others, who have followed the diet, no inherent downward tendency has yet been observed, and progressive improvement seems to be evident in the majority. The longest observation is now only twenty-one months, so the ultimate results are still subject to question. It would not be strange to find that some severe diabetics have too little assimilative function left to maintain life, but thus far it has been possible to avoid such a confession. To some extent the tolerance, but especially the general well-being of the patients, have been improved by the introduction of vigorous exercise,[3] and most of those treated are now earning their living. It is hoped that a real reduction of the mortality of diabetes will be possible; but if ultimate results disappoint this hope, the treatment is justified by the benefit to the comfort and usefulness of the patients. It is in harmony with the observed benefits of carbohydrate restriction and undernutrition in the past history of diabetes, and it makes an efficient application to diabetes of one of the best established principles of all therapeutics, namely, rest of a weakened function. What is further desirable is obviously some positive means of strengthening the weakened function; but no such method has yet been discovered for diabetes.

[2] Allen, F. M.: Prolonged Fasting in Diabetes, Am. Jour. Med. Sc., 1915, cl, 480.

[3] Allen, F. M.: Note Concerning Exercise in the Treatment of Severe Diabetes, Boston Med. and Surg. Jour., 1915, clxxiii, 743.

ABERRANT INTESTINAL PROTOZOAN PARASITES IN THE TURKEY.

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PLATE 48.

During a study of tissues from a series of turkeys which had been included in several experiments designed to throw light on the transmission of the protozoan parasite producing the so called blackhead, the writer came upon a case which furnishes the text for this communication. The history of the animal (No. 98) is given in a recent paper (1).

It was raised with eighteen others in an incubator and brooder. No adult turkeys or poultry had been on the grounds for many years. Nine young turkeys from an infected flock, imported July 8, had been kept confined in a distant enclosure. Hatched about May 15, it was well until July 22, when it appeared quiet, sleepy, and refused food. It was chloroformed in the hope of obtaining fresh tissues for histological examination of the intestines. The autopsy showed an absence of blackhead lesions. There was, however, a distinct increase in mucus in the ceca, and several coccidial cysts, measuring about 24 μ by 17 μ, were found in the feces passed during chloroforming. The contents of the duodenum had a markedly yellowish coloration. The muscular tissue of the body was also tinged yellowish. Subsequent examination of tissues fixed in Zenker's fluid showed the presence of peculiar bodies in the subepithelial tissues of the villi of the small intestine, which were tentatively diagnosed as the asexual or schizogonic cycle of some coccidium. The apparently unusual position of the bodies in the adenoid tissue of the mucosa, where they were found in very large numbers, contrasted with the scarcity of the same within the epithelial layer where they, as will be shown

567

later, were probably not within but between the epithelial cells, induced the writer to give this case a more thorough study.

Coccidia have been generally regarded as exclusively parasites of epithelial cells. The older works of Balbiani (2), von Wasielewski (3), Doflein (4), and others take this for granted. Similarly the investigations by Malassez (5), Labbé (6), Pfeiffer (7), Sjöbring (8), Felsenthal and Stamm (9), Laveran (10), von Wasielewski (11), dealing either with *Eimeria stiedæ* of rabbits or the two known species of avian coccidia (*Eimeria avium* and *Isospora lacazei*) refer only to the epithelial cell parasites.

Metzner (12) was the first to call attention to the presence of coccidia in the subepithelial tissues. He "frequently observed the penetration of *Coccidium cuniculi* into the submucous tissue,[1] regularly into the tunica propria of the cecum, the appendix, and the colon, not infrequently of the small intestine." Metzner promised a discussion of these facts in a subsequent paper which has not, however, appeared.

Fantham (13) mentions the finding of coccidia of grouse in the submucosa, though "much more rarely." He further states that "active schizogony and sporogony go on in the ceca, chiefly in the epithelium, very rarely in the submucosa."

The writer (14), in 1910, described the occasional dislocation of sporonts or gametes of *Eimeria stiedæ* in the rabbit from the epithelial into the subepithelial tissue. This dislocation was ascribed to the transformation of invaded epithelial cells into multinucleated cells followed by disturbance of normal relationship with the adjacent epithelial cells and the subjacent tunica propria. The parasites were not found in the cells of the latter.

Hadley (15) refers to the invasion of the subepithelial tissues of the ceca of turkeys by coccidia. At that time he identified the protozoan parasite of blackhead, which is a purely connective tissue parasite and which does not enter epithelial cells at any time, as a coccidium.

Gérard (16) in describing a coccidiosis of young chicks takes an advanced position on the basis of material studied by him. He states that the schizogonic stage of his parasite goes on in the epithelial and subepithelial tissues and that sporogony goes on only in the epithelium. One of his figures shows many schizonts apparently in the subepithelial cells, but the drawing is evidently not quite true to nature and the large host cells, in certain cases at least, suggest epithelial cells. However, there seems to be enough evidence in his case to indicate extensive

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[1] There seems to be much confusion in the use of the terms mucous and submucous tissue. The writer includes in the mucosa the tissue bounded by the muscularis mucosa. Several authors to be quoted have evidently regarded as submucous the subepithelial portion of the mucosa. Metzner in using tunica propria and submucosa as synonymous is using the latter term in this sense.

invasion of the mucous membrane with a slight simultaneous occupation of the epithelium.

It should be stated here that Rivolta (17), as early as 1873, described coccidia-like cysts in the submucosa of fowls which were relatively large and visible to the naked eye as white points, the size of poppy seeds. In 1878 he (18) again referred to similar white points about 0.5 mm. in size, in the same situation in fowls.

In 1893 the writer (19) published a brief account of similar cysts in the villi of the ileum in calves. These were situated near the free end of the villus, 0.3 to 0.4 mm. in diameter, and filled with mobile and immobile merozoites.

Two genera of sporozoa, very different from one another, yet both connecting the coccidia with the gregarinida, should here be mentioned, Léger and Duboscq's (20) *Selenococcidium* and Tyzzer's (21, 22) *Cryptosporidium*. In the former the schizont is a free-living nematode-like organism entering cells only during the periods of asexual and sexual reproduction. The latter passes its entire life cycle attached to, but not in epithelial cells.

Returning to the case of the young turkey, I wish at present to call attention to certain features only, for the sparse material and the absence of a sexual stage make any definite correlation with earlier work and any interpretation premature.

Sections of the intestinal tract were available from the upper, middle, and lower small intestine and the ceca. Conditions as regards the degree of invasion were practically the same throughout the small intestine, but over lymphoid tissue the parasites were scarce.

The epithelium was still present but lifted off from the subjacent core of the villus. The intervening space was filled with a network of circular lines of a precipitate made up of fine granules. The parasites were visible under a low power as vacuoles arranged in an almost continuous band near the margin of the villus core. Occasional bodies were nearer the central axis of the villus (Fig. 1). Under high powers these vacuole-like bodies were found to be but partially empty. A few were well filled. They consisted of some host cell whose cytoplasm had been moulded into a shell (or ring in section) with the much flattened nucleus against this shell. The contents were a very fine lining membrane within which were roundish bodies of various diameters, 2 μ and more, staining feebly reddish and with or without a mass of chromatin. Frequently a body contained two chromatin masses situated at opposite poles, as if division had taken place. Those bodies which were full of the small spheres, contained

about sixteen or more of more or less uniform size (Fig. 2). The vacuolated appearance under low power was due to the disappearance of some or all of the parasitic contents of the host cell. Prolonged search for the characteristic products of asexual multiplication—falciform bodies—brought to light only two or three parasites containing them. It is not to be denied that these may have been moulded into crescent shape by the pressure of the other growing and segmenting members in the same membrane. One of these crescents is shown in Fig. 3.

In addition to the parasites in the core of the villus, a certain number of bodies were found free in the space between villus core (tunica propria of Stoehr) and epithelium. If, as is claimed by most histologists, this space is an artefact, the free parasites must have been embedded in the adenoid tissue at the base of the epithelial cells and set free when the core was pulled away by the shrinking action of the fixing fluid. These bodies differ from those embedded in the core of the villus in having a more condensed cytoplasm, staining more intensely with eosin. They also contain relatively large and more numerous clumps of a substance staining deeply with nuclear dyes and presumably chromatin (Fig. 1 on the left above). These compact clumps are very irregularly grouped and of various shapes, many of them broadly oval or biconvex. No form which may be regarded as normal was detected, no two being precisely alike.

Relatively few parasites were found embedded in the epithelial layer itself. In the one shown in Fig. 2 there is considerable chromatin flattened against the periphery of the body as if it were a host cell nucleus. If so, the body is in a cell lodged between epithelial cells and not in the epithelial cell itself.

The same parasites were also found, but in relatively small numbers, in the mucous membrane of the ceca.

Assuming for the moment that all the parasites were striving toward the formation of merozoites of characteristic form, the great variation in the size of the products of division and the irregularity as regards chromatin, lead the writer to infer that they were largely degenerating forms. This theory is supported by the abortive attempts at repeated multiplication within the primary cysts and the partial disappearance of their contents.

As stated above, it is impossible from this case alone to determine whether the parasite belongs to one or the other of the two well known species of avian coccidia, or whether it is a foreign, aberrant type which fails to survive in the accidental host. The relative smallness of the schizonts, which measure 10 microns, and of the merozoites (the only one that could be found and measured being 5 μ long) led the writer to assume that it is a foreign species. The species described by Gérard has schizonts up to 30 μ by 42 μ, and merozoites 14 μ long (16).

The location of this parasite within cells of the villus indicates that the former may have actively invaded the cells and that phagocytosis did not play a part. The nature of the cells invaded the writer is unable to state.

In general, the invasion of the substance of the villus is of more than ordinary interest to the pathologist. A relatively large amount of foreign proteid is brought within the reach of the absorbents and the disintegration of the parasites may well account for the symptoms produced. Several other young turkeys died within a week after the case under consideration had been chloroformed, and the writer attributed these deaths to arsenic and lead spray. The symptoms in these cases were somnolence, indifference to food, and diarrhea, and the lesions were confined to hyperemia and swelling of the mucous membrane of the small intestines. Coccidia were not found in the intestinal contents or in scrapings of the mucous membrane. Unfortunately tissues of these cases were not saved for microscopic examination. Similarly there was slight temporary indisposition and drooping among other members of the flock, which may have been due to the unknown parasite. The presence of pheasants, sparrows, and song birds in the territory surrounding our experimental grounds may account for the infection, for the young turkeys were allowed to feed over a certain territory outside of the screened shelter each day (1).

Toward the end of the study of this case, the writer came upon two small areas in sections of the ceca of the same case within which nearly every epithelial cell contained minute protozoa which on further study were diagnosed as true coccidia (Fig. 4). The parasites were in an early stage, either as schizonts or sporonts. Putting all the

facts together, the writer is inclined to regard these epithelial cell parasites as belonging to a species distinct from that in the subepithelial tissue, and perhaps the same as the species represented by the oocysts found in the contents of the large intestine at autopsy. To all appearances we have in these parasites true coccidia to deal with.

The invasion of the subepithelial territory of the mucous membrane raises the question concerning the frequency of this phenomenon among coccidia. Is it that this early stage, coming as it does before or with the earliest symptoms, has been overlooked? The negative evidence the writer has cited above from the writings of others should not count too heavily, for much of the work was done by those for whom the life cycle of the parasite was the chief object of pursuit. It may now be desirable to examine the intestines of young healthy animals and of those in which symptoms are just appearing, to determine to what extent early schizogony goes on in the subepithelial tissues, whence direct invasion of epithelial cells for the sporogony may take place from the base of the cells.

There are so many points of difference between the blackhead parasite and the unknown parasite of the mucous membrane as encountered in this single case, that any attempt to present them would require an elaborate restatement of what is now published. Assuming that they are different, we are confronted with the fact that, even after the blackhead parasite shall have been eliminated, the outlook for raising turkeys without some losses due to avian coccidia and perhaps other still unknown protozoan parasites is not very encouraging. Fortunately the mortality due to these aberrant parasites was low. In any case the specific sources of coccidia and other parasites must be found and dealt with.

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EXPLANATION OF PLATE 48.

FIG. 1. Section through a villus. The main axis is from above down. Only a few nuclei of the epithelium appear on the left below. The core of the villus is surrounded by a bubbly coagulum under the epithelium. On the right, the marginal zone of the villus contains a row of faintly outlined parasites (from above down). The largest, below, appears as a vacuole containing a few remnants of the schizogonic division. On the left margin of the villus near the bottom of the figure several parasites are faintly outlined.

In the subepithelial (bubbly) space on the left, above, a parasite is seen staining more intensely and with six or more clumps of chromatin.

FIG. 2. A parasite within the epithelial layer. The nucleus of the host cell is shown only in a small part in the photograph. The nucleus of an epithelial cell is seen in the invaded territory crowded towards the free border of the epithelial layer. The progeny of the schizogonic (?) division are faintly outlined. Only one shows a nucleus indistinctly.

FIG. 3. A schizont showing what appears to be a merozoite with a nucleus near one extremity. The schizont is situated in the space between the epithelium and the villus core and is evidently attached to the latter. A row of parasites situated in the marginal zone of the villus is faintly indicated.

FIG. 4. Epithelium of one cecum, showing nuclei near the base of the cells and parasites nearer the free border. The parasite farthest to the right contains four nuclei, but only two are in focus.

(Smith: Aberrant Intestinal Protozoan Parasites.)

INDEX TO AUTHORS.

INDEX TO SUBJECTS.